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Award Number: DAMD17-01-1-0248

TITLE: IKK and (Beta)-Catenin in Breast Cancer

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REPORT DATE: July 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual Summary (1 Jul 02-30 Jun 03)

4. TITLE AND SUBTITLE
IKK and (Beta)-Catenin in Breast Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0248

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The Wnt pathway is involved in many differentiation events. Mutations involving downstream components like APC or b-catenin result in nuclear accumulation of b-catenin, which results in cancer. This project has discovered that cytokine, TNFa and ectodysplasin (Eda) and its receptor, Edar regulate b-catenin signaling activity. A conserved sequence, DSGXXS, within the N-terminus of b-catenin and IkappaB, allows for targeted phosphorylation by upstream kinases such as IkappaB kinase. Evidence show that TNFa is able to regulate b-catenin through IkappaB kinase complex. This complex is normally involved in inactivating inhibitor, IkappaB which sequesters NFkappaB in the cytoplasm. The second part of this project shows that NFkappaB signaling is independent of IKK regulation of b-catenin. Wnt signaling is not the only way b-catenin is regulated. Cytokine induced decrease in b-catenin signaling activity is not due to b-catenin degradation but rather, to its re-distribution. This study confirms that there is an 'active' fraction of b-catenin present within the nucleus is responsible for its signaling activity and both TNFa and ectodysplasin reduce or redistribute that fraction.

14. SUBJECT TERMS
No subject terms provided.

15. NUMBER OF PAGES
117

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction: The Wnt signal transduction pathway controls many developmental events in living organisms, including morphogenesis via activation of the β-catenin-TCF/LEF transcription complex. β-catenin is an oncogene which is present in three pools within the cell: 1) at the cell membrane where it is involved in cell adhesion; 2) in the cytoplasm and 3) in the nucleus where it participates in gene transcription [29;58;77]. It is a bi-functional protein and its levels in the cell are normally tightly regulated. However, mutations occurring at the N-terminus of its regulatory domain or in the tumor suppressor, adenomatous polyposis coli (APC) and axin (conductin) genes, prevent effective degradation of β-catenin protein. This leads to its accumulation in the nucleus where it associates with a member of the TCF (T-cell factor) /LEF (Lymphocyte enhancing binding factor) family of transcription factors to activate transcription of downstream target genes such as *c-myc* and *cyclin D1* [57;132]. Inappropriate activation of these genes causes uncontrolled cell growth and finally, cancer. Mutations in β-catenin cause many colon cancers, melanomas and ovarian cancers [19]. In this study, the mechanisms by which β-catenin is regulated are examined to expand the current knowledge and understanding of its role in cancer and development.

The general aim of this project is to develop a better understanding of β-catenin regulation. Early experiments indicated that TNFα treatment was able to repress β-catenin signaling activity. This led to further examination of how this occurred and why. There are two parts to this project; the first is to understand how β-catenin is regulated by TNFα and to study the potential involvement of IKKα and IKKβ. The study of the related family member, ectodysplasin was included because ectodysplasin is involved in morphological skin defects similar to those that are regulated by β-catenin signaling. The second part of this project, involves a study of how β-catenin is repressed by the cytokine pathway and how its outcome in the cells is determined. Previous studies indicate that there may be some "cross-talk" between the Wnt pathway and the TNFα/ NFκB pathway. The aim of this dissertation investigates, in detail, the cytokine regulation of β-catenin signaling.

Body:

I: TNFα Regulates β-catenin Signaling Activity.

Initial experiments to determine if tumor necrosis factor alpha (TNFα) had any effect on β-catenin signaling activity were carried out by treating SW480 colon cancer cells and HEK 293 cells with 20ng/ml TNFα for sixteen hours. SW480 cells have a truncated APC gene and are unable to effectively degrade β-catenin, leading to high endogenous levels of β-catenin signaling. HEK 293 cells do not have high endogenous levels of β-catenin and are useful to rule out the effects that the high β-catenin levels in SW480 cells may have on the observations. To measure β-catenin signaling, a luciferase promoter (Topflash) with four upstream T-cell factor (TCF)/lymphoid enhancing factor (LEF) response elements was used, which allow binding of TCF/LEF transcription factors. The amount of light produced is measured in lumens. A TCF/LEF mutated luciferase reporter (Fopflash) was used as a negative control. The NFκB luciferase reporter has several NFκB binding sites upstream of a thymidine kinase promoter. NFκB reporter was used as a positive control since it is known that it is activated by TNFα (159). Renilla luciferase reporter was used as an internal control to correct for transfection efficiency. All transfections were done in triplicates and repeated at least 3 times after which statistical analysis was performed on the data. In this experiment, SW480 cells were transfected with either Topflash, NFκB or Fopflash reporter for twenty-four hours and treated with TNFα for sixteen hours. The results show that TNFα decreased β-catenin signaling activity by 63% and increased NFκB signaling activity by 187% (**Figure 1A-B**) Since Fopflash reporters have mutated TCF sites and are not able to bind to β-catenin, little signaling was observed. These initial results show that TNFα repress β-catenin signaling activity.

II: Edar Regulates β-catenin Signaling Activity.

The ectodysplasin receptor, Edar was overexpressed by transfecting SW480 colon cancer cells with 50ng Edar and either 0.1ug of Topflash, Fopflash or NFκB reporters. Edar decreased β-catenin signaling activity by 87% and significantly increased the NFκB signaling activity by about 200% (**Figure 2A**). Similar experiments were performed with the ligand, Eda-A1 and found that it was also able to slightly decrease β-catenin signaling by 26%. This was

somewhat surprising since the expression of the Eda receptor, Edar has previously been reported only in the skin (**Figure 2B**). It is expected that the overexpression of Edar would result in a larger repression compared to the overexpression of the ligand itself since the number of receptors per cell may be a limiting factor and addition of the ligand alone would result in a smaller degree of activation. Similarly, the increase in NFκB activity only increased by 133%. In the presence of Edar, overexpression of the ligand results in a constitutively active system that stimulates downstream proteins after Eda-A1 is able to bind to its receptor, Edar. Because Eda-A1 was able to repress β-catenin signaling in SW480 cells, SW480 cells were examined to see if they expressed Edar. Similar experiments had been repeated in HEK 293 cells and so we also harvested RNA from these cells to determine if they had endogenous expression of Edar. A forward primer from position 1129 and a reverse primer from position 1725 was used and performed 1-step RT-PCR. A band of 597 bp in size was expected, which corresponds to Edar. Our results confirmed that SW480 cells and to a much lesser extent, HEK293 cells do express the receptor (**Figure 3**).

III: β-catenin and cyclin D1 are localized in the nuclei of epidermal cells in IKKα (-/-) mouse (Experiments were performed by Christy Jarrett)

A number of studies indicate that IkappaB Kinase (IKK)α does not play a dominant role in the regulation of NFκB signaling [5;23]. Although the IKKα (-/-) mouse does not have a marked NFκB phenotype, it does exhibit a thickening of the epidermis and has a defect in keratinocyte differentiation [12;30]. Moreover, Hu et. al. had shown that the role of IKKα in keratinocyte differentiation is not exerted through IKK activation of NFκB [13]. Because elevated β-catenin signaling is associated with hyper-proliferation of epidermal basal cells, and the Wnt/β-catenin/TCF pathway is implicated in epithelial stem cell proliferation and differentiation, β-catenin localization in the epidermis of IKKα (-/-) mice was investigated. Unfortunately, the de-phosphorylated β-catenin antibody did not work on paraffin sections. For these experiments, a β-catenin antibody that detected the C-terminal of β-catenin protein was used. Membrane β-catenin staining was observed in both wildtype and IKKα (-/-) epidermis but a marked increase in the number of cells with

nuclear β-catenin occurred throughout the epidermis and particularly in the basal region of IKKα (-/-) mice. (**Figure 4A-D**) Very few cells in the normal epidermis exhibited nuclear β-catenin staining at this stage in development. Nuclear β-catenin staining also occurs in human epidermal keratinocytes expressing N-terminal deleted form of β-catenin but not in normal keratinocytes or in normal human skin [35]. Localization of β-catenin in the nucleus suggests an increase in β-catenin/TCF signaling. Thus β-catenin target gene cyclin D1 was localized. Like β-catenin, cyclin D1 was markedly upregulated in the basal regions of the IKKα (-/-) mouse epidermis. (**Figure 4E-H**) This indicates that β-catenin and IKKα may have some interaction.

IV: Constitutively Active IKKα and β further down-regulate β-catenin Signaling Activity and Augment NFκB Signaling Activity.

Previous sections have shown that TNFα and Eda/Edar repress β-catenin signaling. Further evidence has also been shown that β-catenin nuclear staining is up-regulated in IKKα (-/-) fibroblast cells. This leads to examine if the individual IKK subunits, IKKα and IKKβ are involved in TNFα and Eda/Edar repression β-catenin signaling activity. To determine whether TNFα could regulate β-catenin signaling through the IKK complex, SW480 colon cancer cells were transfected with constitutively active IKK mutants. These constitutively active mutants (CA IKKα and CA IKKβ were generated by changing serine residues within the conserved region of the activation loop at position 176 and 180 in IKKα and 177 and 181 within IKKβ to glutamate) (SS to EE). This mutation results in a conformational change that is similar to its activated state when it is phosphorylated. SW480 cells were a good model to use because it has a truncated APC gene which prevents β-catenin from being degraded, resulting in a high level of endogenous β-catenin in the cell. As described above, reporters, Topflash, Fopflash or NFκB were used to measure β-catenin signaling activity. Again, experiments were done in triplicate and each experiment was repeated at least three times. TNFα reduced β-catenin signaling as shown before and CA IKKα and CA IKKβ by themselves also decreased β-catenin signaling by 82% and 90% respectively. As expected, the presence of both TNFα and CA IKK mutants further decreased its signaling by

5% for IKKα and 3% for IKKβ. It also further augmented NFκB signaling activity after treatment with TNFα by more than 2 fold confirming its role in activation of NFκB signaling (**Figure 5**). This suggests that IKK may have role in regulating β-catenin activity. Since CA IKK mutants decrease Topflash activity, the next logical step was to determine if dominant negative IKK mutants could reverse this effect.

V: Dominant Negative IKKα and β reverse TNFα and Edar down-regulation of β-catenin Signaling Activity and block NFκB Signaling Activity.

To further determine the contribution of the IKK complex in downstream events following TNFα stimulation, dominant negative IKK mutants (doses ranging from 0.1ug to 1.0ug) were transfected into the same cell lines together with Topflash, Fopflash or NFκB reporters. Experiments using cyclin D1-luciferase reporter in SW480 cells were repeated to show that observations are dependent on TCF binding elements, which are downstream targets of β-catenin. This cyclin D1 promoter construct (-163CD1Luc) has TCF/LEF sites as well as CREB, EGR1, SP1, E2F1 and NFκB binding sites (44, 66). The dominant negative mutants (DN IKKα and DN IKKβ) were generated by changing serine residues at position 176 and 180 to alanine in IKKα and 177 and 181 to alanine in IKKβ (SS to AA). This mutation prevents kinase activation in response to TNFα stimulation. Both DN IKKα and DN IKKβ were able to reverse TNFα down-regulation of β-catenin signaling in a dose-dependent manner (**Figure 6A**). Both dominant negative mutants were also able to block any increase in NFκB signaling activity after treatment with TNFα. DN IKKα was able to further increase Topflash signaling activity at its highest dose, unlike DN IKKβ. This may be a result of over-expressing large amounts of exogenous DNA in the cell system resulting in an over-stimulation effect. SW480 cells, as mentioned earlier, are unable to degrade β-catenin as a result of a mutated APC gene. Thus APC was added back to determine if this had any effect on the observations (**Figure 6B**). As expected, APC decreased β-catenin signaling by 83%, however, neither of the dominant negative forms of IKK were able to reverse the inhibition of β-catenin signaling activity. In addition, APC did not potentiate or inhibit the ability of TNFα to regulate NFκB activity in SW480 cells. These data strongly suggest that the effects of IKK are independent of APC. These experiments were

repeated in 293 cells, which have less endogenous β-catenin than SW480 cells as a result of an intact APC gene, with similar results (**Figure 6C**). This strongly indicates that both IKKα and IKKβ are involved in cytokine regulation of β-catenin signaling but are not required to mediate APC effects.

A similar experiment with Eda/Edar was also performed. Both DN IKKα and DN IKKβ were able to partially reverse Edar down-regulation of β-catenin signaling in a dose-dependent manner. (**Figure 7A**) In contrast to TNFα, the highest dose of DN IKK was unable to fully reverse the repressive effects of Edar; DN IKKα was only able to reverse repression by 73% and DN IKKβ was only able to reverse repression by 51%. This may be a result of over-stimulating the system because of the addition of large amounts of exogenous DNA. The cyclin D1 promoter was also used to confirm that the observations are independent of reporter activity since cyclin D1 is a downstream target of β-catenin, and similar results were observed. Again, 1.0 ug of DN IKKα was able to reverse repression by 62% and a similar dose of DN IKKβ was able to reverse repression by 65% (**Figure 7B**) It is interesting to note that the cyclin-D1 promoter with the TCF site mutated had increased activity with the over-expression of Edar. As mentioned above, there are other binding sites within the cyclin D1 promoter, such as CREB, NFκB and Sp1 sites. It is very likely that the increase in cyclin D1 promoter activity in the absence of TCF/LEF binding sites is due to NFκB activation. Another possible explanation may be that exogenous expression of Edar results in the removal of negative regulators that normally bind to the promoter resulting in an increase in response to Edar. Additional experiments were also performed by over-expressing Eda-A1. DN IKKα was able to reverse Eda repression of β-catenin signaling. Only a partial reversal of 83% was observed with DN IKKβ (**Figure 7C**). It is unclear why similar amounts of DN IKKβ are unable to reverse the repressive effects to a similar degree as DN IKKα. Again, over-expression assays could result in an over-stimulation of the cell system and IKKβ may have additional pathways downstream of Edar that is activated, which makes it difficult for DN IKKβ to block. Further experiments were repeated in another colon cancer cell, Caco2 cells, to show that these effects are independent of cell lines. Again, Edar repressive effects

on β-catenin signaling activity was reversed at the highest dose of dominant negative IKK mutants. As expected, Edar increased NFκB activity by 44% and this was blocked after the addition of the IKK mutants (**Figure 7D**). Similar to TNFα results, the data strongly suggests that both IKKα and IKKβ are involved in Edar regulation of β-catenin signaling. However, because of the limitations of over-expression studies, another method to provide evidence of the involvement of IKK was utilized.

VI: TNFα repression of β-catenin Signaling Activity is Abolished by IKK RNAi and in IKK (-/-) mouse embryonic fibroblast cells.

Over-expression of IKK through transfection of exogenous plasmids, although the easiest and the fastest way to determine its contribution in the pathway, is not the best method. The addition of large amounts of DNA may affect the stoichiometry of the IKK complex within the cell and this may cause unknown effects within the signaling pathway, which lead to misleading observations. Gene silencing technique was used to determine the importance of IKK in both TNFα and Edar down-regulation of β-catenin signaling activity. In gene silencing, double-stranded RNA (dsRNA) corresponding to IKKα or IKKβ is added to cells and rapid degradation of mRNA silences its expression within the cell. Appropriate dose was determined to silence each of the IKKs in SW480 cells through western analysis (**Figure 8A**) and treated cells with RNA oligonucleotides against IKKα and IKKβ. Repression of β-catenin signaling by TNFα was markedly inhibited in the presence of IKKα or IKKβ RNAi (**Figure 8B-C**). Both IKKα and IKKβ were further examined to determine their involvement in this pathway. We know that RNA interference has its shortcomings, since it is transient and there is increasing evidence that there may be non-specific inhibition and toxicity to cells. E.g. IKKβ RNA at low doses, did not silence IKKβ in SW480 cells. The use of the RNAi at doses higher than 100nM resulted in cell death. Targets were also chosen at sites other than the current RNAi for IKKβ, however, it was also unsuccessful in knockout out specific gene expression. Since there was much technical difficulties in manipulation of the systems to prevent cell toxicity at high doses of RNAi, IKKα (-/-), IKKβ (-/-) and double knockout mouse embryonic fibroblasts (MEFs) were used to show that IKK is involved in TNFα and Eda/Edar regulation of β-catenin signaling. MEF cells were

treated with TNFα for 16 hours after transfecting them with Topflash or NFκB reporters as described previously, and compared them with normal parental MEF cells. Both the IKKα (-/-) and IKKβ (-/-) cell lines were insensitive to TNFα repression of β-catenin activity when compared to the wild type. (**Figure 9**) NFκB activity in TNFα treated IKKα (-/-) and IKKβ (-/-) cell lines was reduced compared to wild type cells. This confirms that the IKK complex plays a regulatory role in TNFα down-regulation of β-catenin signaling activity. To further confirm the role of IKK, wild type IKKα and IKKβ were over-expressed in the knockout mouse embryonic cells and treated them with TNFα. As expected, addition of either of the IKKs restored TNFα-mediated repression of Topflash reporter activity and that addition of both IKKs to the double knockout cells decreased TNFα repression of β-catenin signaling almost 10 fold. (**Figure 10A-C**) Addition of both IKKs also augmented NFκB activity almost 2 fold. (**Figure 10D-F**)

Experiments were repeated by over-expressing Edar. (**Figure 11A-F**) Contrary to earlier results with dominant negative IKK mutants, the knockout MEF cells showed a similar repression in signaling activity even when either IKKα or IKKβ were absent. (**Figure 12**) However, when both IKKα and IKKβ were removed in the double knockout MEFs, significant absence of TNFα repression was observed. (**Figure 12**) As explained earlier, the use of over-expression studies results in over-stimulation of the cell system. RNAi data were not perfect since there were difficulties in silencing IKKβ without resulting in cell toxicity. MEF cells, on the other hand are the best system to use since the gene is already absent. Thus taking into account these data, there is a strong indication that both IKKα and IKKβ are required to transduce the effects of Edar on β-catenin signaling activity and further confirms that both IKKα and IKKβ are required to mediate Edar regulation of β-catenin signaling.

VII: Influence of NFκB activity on TNFα/ EDAR repression of β-catenin Signaling.

As discussed above, phosphorylation of the consensus sequence, DSGXXS, found in both β-catenin and IκB stimulates their interactions with the ubiquitin ligase β-TrCP leading to their degradation via the ubiquitin-proteasome pathway

[227-229]. One study has shown that the β-catenin/TCF complex increases β-TrCP levels by a post-transcriptional mechanism to result in a decrease of β-catenin and increase in NFκB activity [230]. In addition, disruption of either murine GSK-3β and IKKβ genes result in embryonic lethality due to hepatic apoptosis from increased sensitivity to TNFα [76]. These results suggest potential relationships between β-catenin and NFκB signaling pathways. Activation of NFκB signaling was examined to determine if it may indirectly be responsible for the cytokine repression of β-catenin activity. 80nM of RNA oligonucleotides against p65 subunit of NFκB were transfected into SW480 cells to silence the transcriptionally active form of NFκB i.e. p65. Confirmation that the gene was silenced was determined through western analysis of protein lysates after transfection and by luciferase assays using the NFκB reporter. In (**Figure 13A-C**), neither TNFα nor Edar activated NFκB signaling activity in the presence of p65 RNAi (**Figure 13D**). Despite the absence of NFκB, β-catenin signaling was still repressed by 34% after treatment with 20ng/ml of TNFα, while over-expression of Edar inhibited β-catenin signaling by 51%, showing that NFκB does not play a role in TNFα or Edar repression of β-catenin signaling (**Figure 13B,D**). This indicates that cytokine repression of β-catenin activation is independent of the activation of NFκB signaling and that in our system, these 2 pathways are independent of each other. It is very likely that the repression of β-catenin signaling is a result of an increase in degradation of the protein.

VIII: Involvement of β-TrCP in Down-regulating β-catenin Signaling

Previous data has ruled out a role for NFκB activation on the repression of β-catenin signaling activity. Attention was next focused on the possibility of increased degradation of β-catenin. Thus the possibility that an increase in β-catenin degradation as a result of its phosphorylation by the IKK complex was examined. Since it is well-known that β-TrCP is the F-box protein involved in the SCF ligase complex responsible for the degradation of β-catenin in the canonical Wnt pathway, an investigation was done to determine if this protein was involved in the TNFα and/ or Edar effects. Specifically, the hypothesis that

TNFα and Edar inhibit β-catenin signaling through increased degradation of the protein as a result of targeted phosphorylation of β-catenin and recruitment of β-TrCP and subsequent ubiquitination and proteosomal degradation was tested. Luciferase assays and similar transfections on HEK 293 cells were performed. HEK293 cells have lower endogenous β-catenin and they were transfected with either dominant negative mutant β-TrCP1 or β-TrCP2 (DN β-TrCP) and treated with 20ng/ml of TNFα or transfected with 50ng of Edar (**Figure 14A, 15A**). As expected, when dominant negative mutant β-TrCP1 was present alone, there was a 23 fold increase in β-catenin signaling activity since β-catenin degradation is blocked. However, even with this marked increase in signaling activity, DN β-TrCP1 did not completely reverse TNFα or Edar repression of β-catenin signaling. There was still a 31% decrease in β-catenin signaling when TNFα was added and a 74% decrease when Edar was overexpressed as compared to their controls. Thus β-catenin signaling was still significantly repressed when either TNFα or Edar was present, indicating that β-TrCP-mediated ubiquitination is only partly responsible for the inhibition in β-catenin signaling. Similar experiments with their controls were performed using NFκB reporter showing no significant induction of luciferase activity after treatment with TNFα or overexpression of Edar in the presence of DN β-TrCP1 (**Figure 14B, 15B**). This indicates that the DN mutant is blocking cytokine activation of NFκB activity as one would expect. It is surprising to observe that DN β-TrCP2 does not prevent NFκB activation after the addition of TNFα or Edar. It is possible that DN β-TrCP2 does not work as well as the dominant negative β-TrCP1. It is also possible that the cells do not express this F-box protein and thus are insensitive to the addition of a dominant negative mutant. Since antibodies are not commercially available, 1-step RT-PCR was utilized to determine the expression of the gene in the cell lines that the experiments were performed in, i.e. SW480 and HEK293 cells. Both of these F-box proteins are expressed in both cell lines ruling out the second explanation (**Figure 16A**). A schematic of the location of the primers used to detect the gene is shown in **Figure 16B**. Furthermore, the role of β-TrCP2 is not clear. It is interesting to note that even though β-TrCP1 and β-TrCP2 share 78% amino acid sequence homology, both are found on different chromosomes; β-TrCP1

is found on 10q24-25 and β-TrCP2 is found on 5q33-34 [231]. It is possible that even though both isoforms may share similar roles in ubiquitinating IκBα, β-TrCP2 may have additional functions within the cell that involve other pathways. However, the data here indicates that β-TrCP1 may be involved in degrading β-catenin protein after stimulation with TNFα or Edar but by itself, is not responsible for the decrease in signaling observed. There strongly indicates that a relocalization of β-catenin must occur, most likely from the nucleus to the cytoplasm or membrane.

IX: TNFα/ Edar Decreases Nuclear De-phosphorylated β-catenin

Previous sections have already ruled out NFκB activation in the repression of β-catenin signaling activity and degradation via the F-box protein, β-TrCP that is also involved in β-catenin degradation within the canonical Wnt pathway. Re-localization of β-catenin out of the nucleus is another possible explanation of the inhibition in its signaling activity. Earlier data generated from the Byers lab have shown that phosphorylation of certain serine residues in the N-terminal of β-catenin can regulate its stability by targeting it for ubiquitination [195]. Although a putative IKK consensus sequence is present in the N-terminal region of β-catenin and is a likely target for IKK, mapping studies indicated that several other regions of β-catenin can serve as substrates for IKK [195]. It is also possible that IKK may affect β-catenin mediated trans-activation by phosphorylating another component of the transcriptional machinery. If TNFα and Edar or activation of IKK regulates β-catenin signaling by targeting it for ubiquitination and protein degradation, one would expect that like APC, axin or activated GSK-3β, they would affect β-catenin protein levels [232;233]. If TNFα/IKK and Edar phosphorylation of β-catenin does not alter its degradation, it was hypothesized that it might alter the localization of the de-phosphorylated "active" form of β-catenin. Immunocytochemistry was used to test this hypothesis. Double-labeling studies of β-catenin in SW480 cells transiently transfected with CA IKKs revealed no significant alteration in total β-catenin protein or localization in the cells expressing IKKs when compared to control cells. (**Figure 17A-C**) Recent papers by Clevers et al. have shown that level of an 'active' form of β-catenin

is increased following Wnt stimulation [234]. Since there were no changes in β-catenin protein localization when probed with the C-terminus β-catenin antibody, an investigation was done to confirm if the changes in signaling activity may be due to changes in distribution of this 'active' fraction of β-catenin. CA IKK mutants were transfected into SW480 cells or treated with either TNFα or Edar and the 'de-phosphorylated' β-catenin, α-ABC antibody was used to probe for this 'active' fraction. Immunocytochemistry shows that there is a marked decrease in nuclear staining after treatment with TNFα (**Figure 18A-B**) Similar results were obtained with the transfection of Edar (**Figure 19A-C**) or the overexpression of CA IKK mutants (**Figure 20A-C**). Data was quantitated and the number of cells with altered localization of phosphorylated β-catenin was determined by scoring 10 fields of view (**Figure 18C**). Thus no nuclear staining was observed with cells expressing Edar or CA IKKα or CA IKKβ indicating that either the de-phosphorylated form of β-catenin is degraded or relocalized out into the cytoplasm to be degraded. Note that this quantitation does not take into account cells with reduced but still detectable nuclear localization of activated β-catenin after treatment with TNFα and therefore represent an underestimate of the effects of TNFα. There was a significant number of cells which did not have any nuclear staining of the de-phosphorylated β-catenin. This 'activated' β-catenin was almost always absent from the nuclei of cells transiently transfected with CA IKK α or CA IKKβ. (**Figure 20A-C**) In cells with CA IKKα expressed, only 12% of cells had nuclear staining and in cells with CA IKKβ expressed, only 6% of cells had nuclear staining. Not all cells have similar amounts of endogenous β-catenin present and the presence of CA IKK mutants may only be able to decrease a certain amount of β-catenin within the cell. Cells with more endogenous β-catenin may have 'residue' levels left which are detected by the antibody. These results confirm that the effects of TNFα and Edar on β-catenin signaling are not associated with degradation of total β-catenin protein but do involve changes in the localization of the 'activated' β-catenin fraction. Furthermore, this also confirms that there is a pool of β-catenin that is regulated by TNFα and Edar within the nucleus and this pool is responsible for the decrease in

reporter activity. The results here suggest that the N-terminus of β-catenin is regulated by activated IKKs through phosphorylation of specific residues. In the next experiments, western analysis of the de-phosphorylated β-catenin antibody was used to detect any changes in protein expression of this 'active' fraction within the cell.

X: TNFα / Edar Do Not Change Total β-catenin Protein Levels

Since completely blocking TNFα/Edar repression of β-catenin signaling by inhibiting β-TrCP was unsuccessful, experiments were repeated using western analysis to examine protein levels of β-catenin in the cytoplasm after treatment with TNFα. A time course was set up to 'catch' the gradual decrease in protein expression levels over time. However, the western blot analysis showed that when SW480 cells were treated with 20ng/ml of TNFα over sixteen hours, there was no change in total β-catenin levels (**Figure 21A**). Similar results were obtained in HEK 293 cells (**Figure 21B**). Since IKK can phosphorylate β-catenin, phosphorylated forms of β-catenin was examined by using different phospho-specific β-catenin antibodies to probe cytoplasmic fractions of SW480 treated with TNFα. Although total β-catenin levels did not change, we found that β-catenin phosphorylated at residues Ser33, 37 and Thr41, increased after treatment with TNFα for 30min and decreased to background levels (**Figure 22**). The data suggest that TNFα treatment results in a transient phosphorylation at the N-terminal serine and threonine residues but since there is no decrease in total β-catenin protein levels, this phosphorylation does not target cytoplasmic β-catenin for degradation .

XI: TNFα repression of β-catenin activity involves phosphorylation of N-terminal serine and threonine residues:

Immunocytochemistry results indicate that TNFα and Edar might be regulating β-catenin at certain residues on the N-terminus. If so, then deletion constructs of β-catenin which do not have the amino terminus should be resistant to the effect of TNFα on β-catenin signaling activity. Deletion constructs were made by removing the N-terminus (1-142aa) or the C-terminus (623-781aa) of β-catenin and transfecting these constructs into wild type MEF cells (**Figure 23A**). Removal of the amino and carboxyl terminus made the cells resistant to the effects of TNFα repression of β-catenin

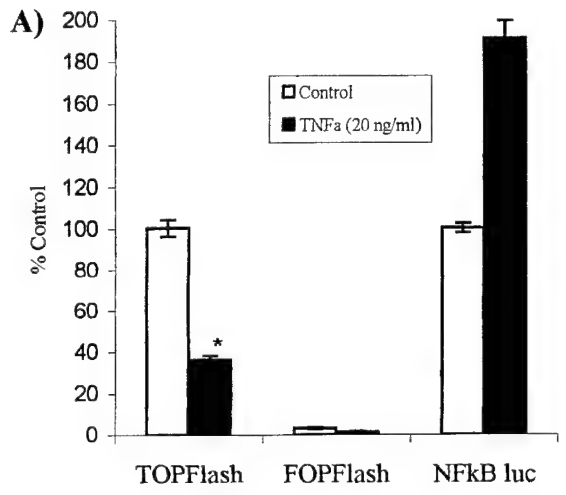
signaling as expected, indicating that these domains play an important role in TNFα regulation of β-catenin signaling. Similar experiments were repeated by over-expressing Edar and were surprised to observe that there was a repression of β-catenin signaling activity in all deletion mutants (**Figure 23B**). The C-terminus is the transactivation domain, which is responsible for its transcriptional activity and removal of this region results in low reporter activity of β-catenin. It is not surprising to observe that treatment with recombinant TNFα is unable to repress β-catenin signaling further since the intrinsic activity of the deletion mutant is too low to start with.

Since cells became resistant to the effects of TNFα after removal of the N-terminus, a further examination was done to determine if there was any change in localization of β-catenin phosphorylated at the N-terminus within the cell after treatment with TNFα. SW480 cells were stained with antibodies that recognize β-catenin only when it is phosphorylated on serines 41 and/or 45. No staining was observed in control cells but a dramatic increase in nuclear staining was observed following treatment with TNFα (**Figure 24A-B**). Treatment of SW480 cells with 20ng/ml of TNFα also resulted in an increase in staining using an antibody specific for β-catenin when it is phosphorylated on serines 33, 37 and threonine 41. This staining pattern was cytoplasmic/membrane rather than nuclear (**Figure 24C-D**). Similar results were observed in HEK293 cells (not shown). These experiments were performed over two hours and the most obvious difference was observed at thirty minutes (other data not shown). The data here indicate that one or more of the N-terminal serine residues are important for TNFα to repress β-catenin signaling activity. The exact residues involved are unknown but earlier data indicate that phosphorylation sites that target β-catenin for degradation (Ser 33,37, 45 and Thr41) are probably involved. To formally test this hypothesis, mouse embryonic fibroblasts cells (MEFs) were transfected with β-catenin, mutated either on residues Ser 33, 37, Thr 41 and Ser45 or on serine 45 residue alone. Expression of β-catenin in wild type MEFs was repressed by 55% after treatment with TNFα for sixteen hours (**Figure 25A**). In contrast, wild type MEFs transfected with β-catenin mutated on 33, 37, 41 and 45 or on serine 45 alone were resistant to repression by TNFα (**Figure 25B-C**). It is interesting to note that the

signaling activity increases with the presence of mutated β-catenin after treatment with TNFα. It is possible that that there may be co-repressors that bind to β-catenin and are responsible for the repressive effects of TNFα on β-catenin signaling. Mutations on these sites prevent the co-repressor from binding and subsequently inhibiting β-catenin signaling. Taken together these data suggest that serine 45 is a key residue in the regulation of β-catenin repression by TNFα and activated IKK. To specifically test this, another colon cancer cell line, HCT116, which has normal APC but expresses one allele of β-catenin in which serine 45 is deleted and one normal allele, was used. Initial experiments confirmed the ability of TNFα and CAIKKs to down-regulate β-catenin signaling activity in parental HCT116 cells (**Figure 26**). Another form of HCT116 cells in which either the normal or the mutated β-catenin allele had been removed by somatic cell knockout was used [235]. Remarkably, cells in which the normal allele had been removed (only expressing mutant β-catenin) were resistant to the effects of TNFα whereas the parental cell line and cells in which the mutant allele was deleted (only expressing normal β-catenin) were sensitive (**Figure 27**). Edar was overexpressed in wild type MEF cells together with different β-catenin point mutants. There was a 50% decrease in signaling activity with wild type β-catenin. Presence of β-catenin mutated at Ser45 also showed a decrease of 49% after Edar overexpression (**Figure 28**). However, when all four phosphorylation sites are mutated, the cells become more resistant to the effects of Edar. Unlike earlier findings with TNFα, a single mutation of Ser45 residue did not make any difference to the repressive effects of Edar on β-catenin signaling. It seemed to indicate that IKK was the only common point between TNFα and Edar repression of β-catenin signaling activity since Edar does not seem to behave in a similar manner to TNFα. A recent paper by Chaudhary et al. showed that the death domain of Edar is required to activate NFκB, JNK, and caspase-independent cell death pathways and that these activities are impaired in mutants lacking its death domain or those associated with anhidrotic ectodermal dysplasia and the downless phenotype [169]. A schematic of the different Edar deletion constructs, including the wild type is shown in **Figure 29A.** **Figure 29B** shows the activity of these mutants on NFκB reporter activity. Another approach was taken to determine

if the death domain of Edar may be involved in regulating Edar repression on β-catenin signaling (**Figure 29C**). Results indicate that the region within the intracellular domain of Edar that includes the death domain (amino acid 225-448), is required to repress β-catenin signaling activity. It is interesting that to note that autosomal recessive mutation, E379K has little NFκB activity and still represses β-catenin signaling. This confirms the independence of NFκB and β-catenin signaling pathways. Also, the autosomal dominant mutation, R420Q still has significant β-catenin signaling activity and yet both point mutations produce similar phenotypes, indicating that possibility that β-catenin signaling is not involved in ectodermal dysplasia. However, more deletion mutants and sequence analysis would have to be done in order to determine the exact region required for repressing β-catenin signaling.

Figure 1: Effect of TNFα on β-catenin signaling. A) SW480 cells and B) HEK293 cells were transfected with 0.1ug of reporter, Topflash or NFκB and incubated for twenty-four hours. The cells were then treated with 20ng/ml of TNFα for a further sixteen hours. Cells were harvested and the luciferase activity assayed. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$.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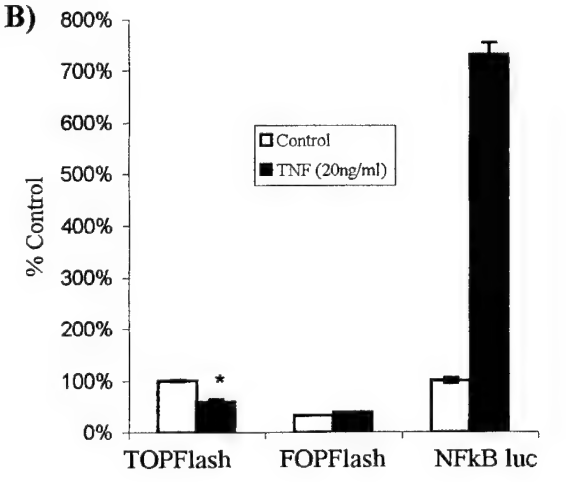
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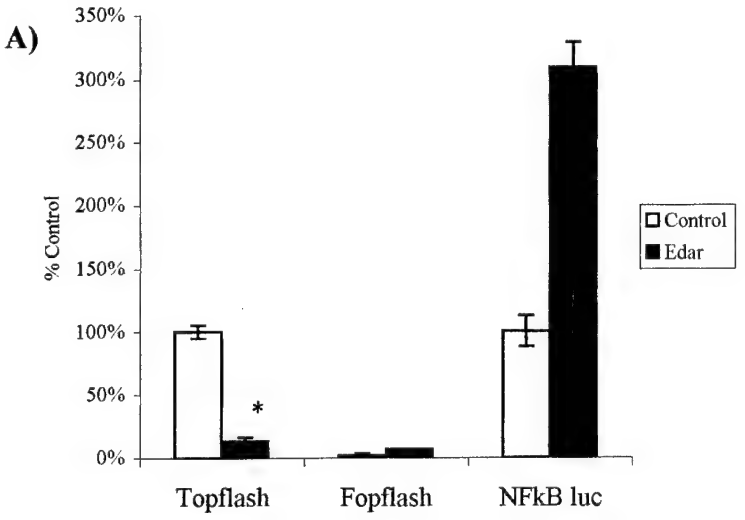
Figure 2: Effect of Eda/Edar on β-catenin signaling. A) SW480 cells were transfected with 50ng of ectodysplasin receptor, Edar together with 0.1ug of either Topflash or NFκB reporter and allowed to incubate for twenty-four hours. B) SW480 cells were transfected with 50ng Eda and a similar amount of reporters for twenty-four hours. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test.* denotes statistical significance where $p < 0.05$.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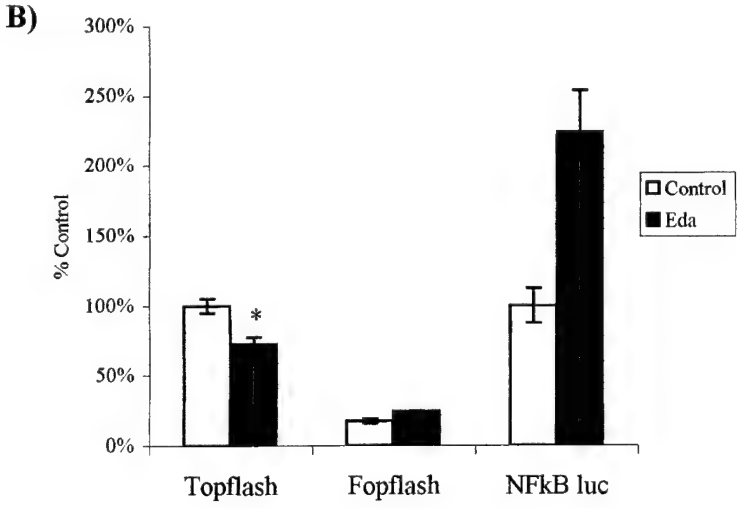
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Figure 3: Expression of Edar in SW480 and HEK293 cells. RNA was harvested from 2 cell types, SW480 and HEK293 cells as described in Materials and Methods section. One-step PCR was performed and the product was run on 2% agarose gel together with 100bp molecular weight marker. A band corresponding to Edar is observed on the gel at around 600bp and is indicated by arrow. There is another unknown band about 400bp in size.

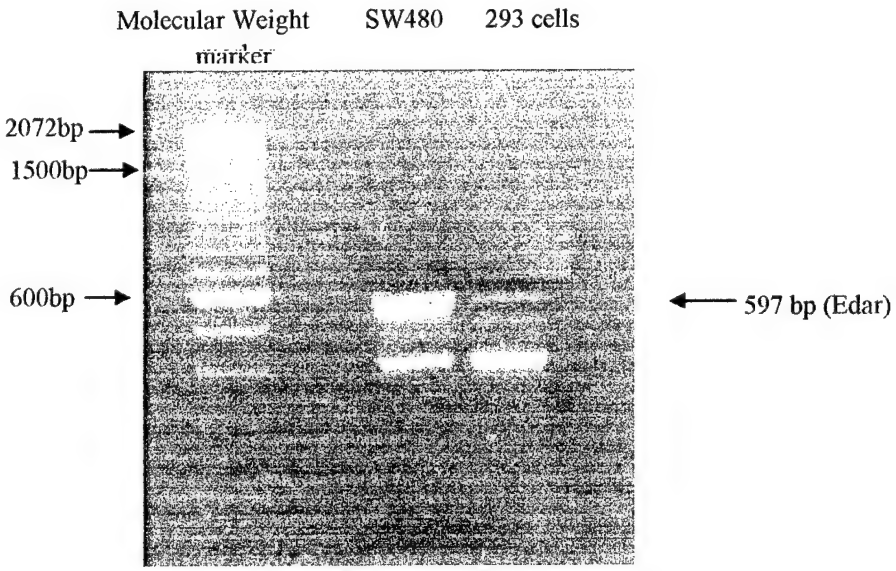
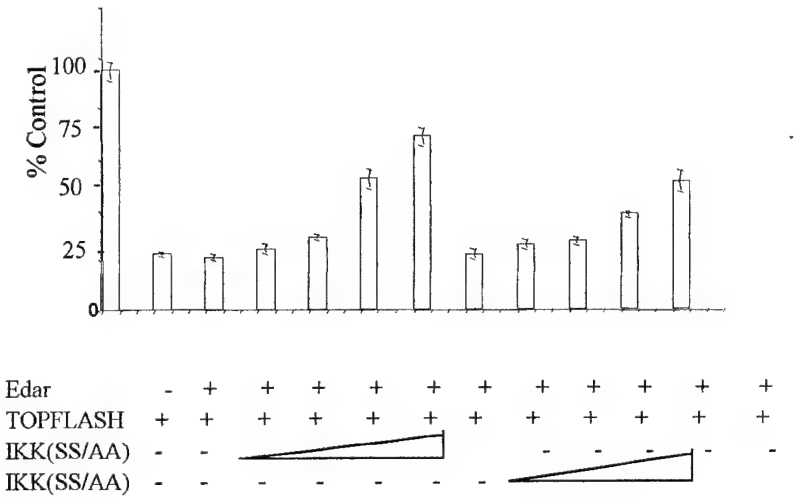

Molecular Weight marker
SW480
293 cells
2072bp
1500bp
600bp
597 bp (Edar)

Figure 4: β-catenin and cyclin D1 are localized in the nuclei of epidermal cells in IKKα -/- mouse. Both wildtype (WT) (A) and IKKα-/- (B) embryos were stained with mouse IgG and hematoxylin. No background staining was observed with the IgG control antibody. Note the thickened epidermis of the IKKα-/- embryo. Because hematoxylin masked the nuclear β–catenin staining, the embryos were stained with β–catenin alone. Membrane staining was observed in both WT (C) and IKKα-/- (D). However, an increased number of epidermal cells with nuclear β–catenin (arrows) was observed in the IKKα-/- mouse. Cyclin D1 expression was also increased in the basal aspects of the epidermis of the IKKα-/- mouse. (E-H) Cyclin D1 staining was performed in Richard Pestell's laboratory

A)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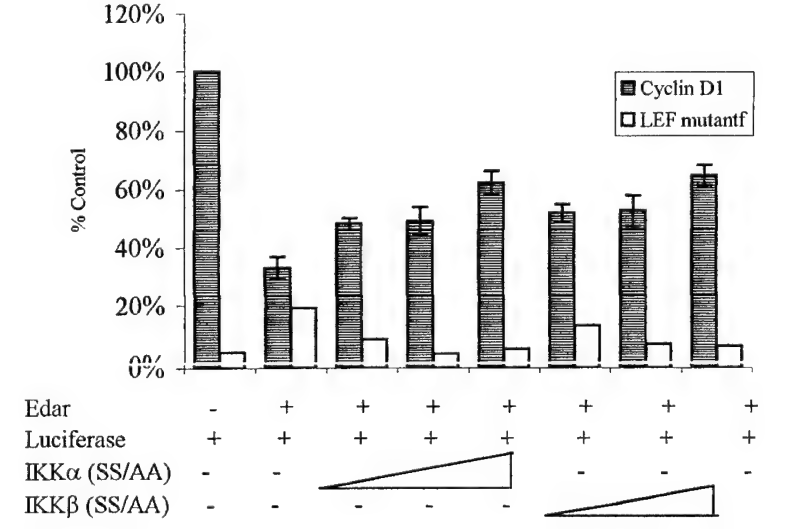
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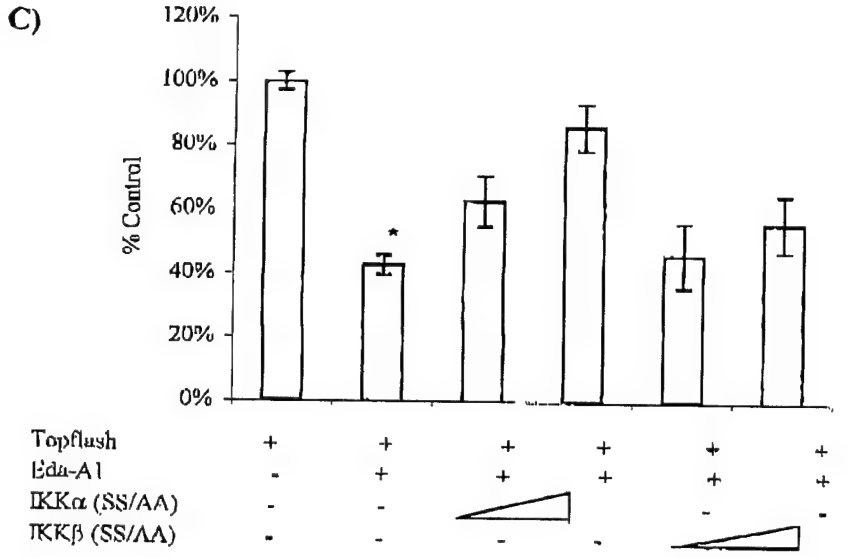
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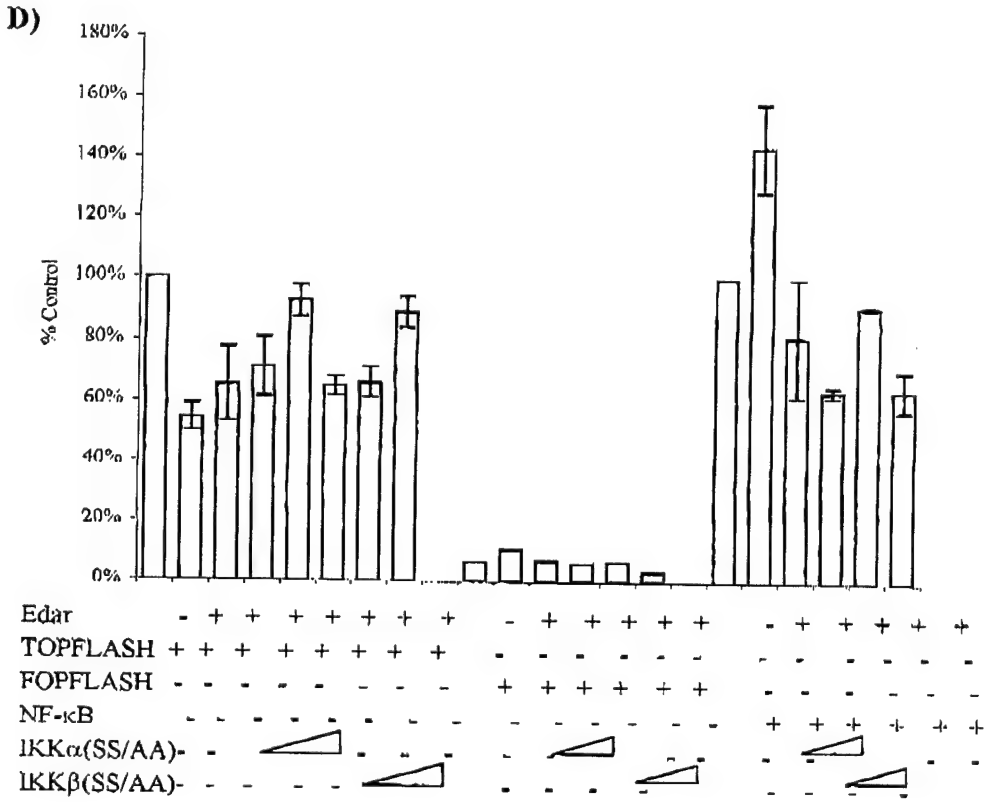
IKKβ (SS/AA) - - - -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IKKα(SS/AA)- - - - - - - - - - - - - - - - - - - -
IKKβ(SS/AA)- - - - - - - - - - - - - - - - - - - -

Figure 5: Effect of constitutively active IKKα and IKKβ mutants on β-catenin signaling. SW480 cells were transfected with either 0.1ug of Topflash or NFκB reporter and 0.5ug of constitutively active IKK mutants. After twenty four hours, they were treated with 20ng/ml of TNFα for a further sixteen hours and harvested. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$.

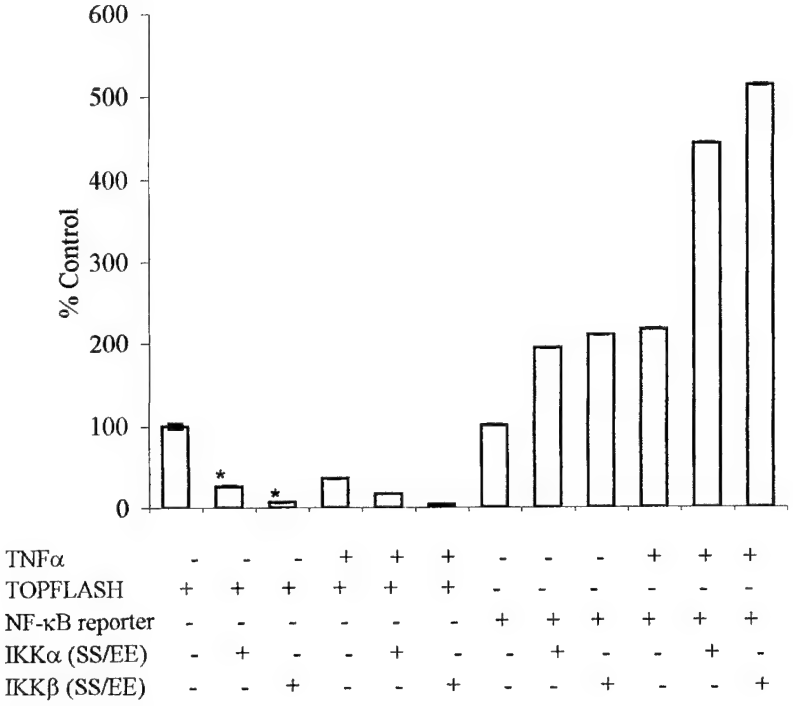
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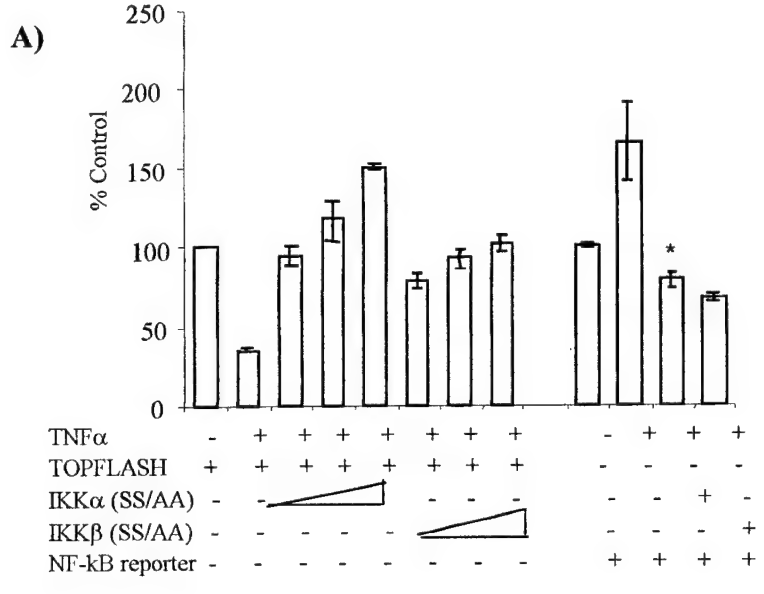
Figure 6: Effect of dominant negative IKK mutants (DN-IKK) and APC on β-catenin signaling. A) SW480 cells were transfected with 0.1ug of Topflash reporter and 0.1ug, 0.5ug and 1.0ug of DN-IKK mutants. When NFκB was used, 1.0ug of DN-IKK mutants were used. After twenty-four hours, cells were treated with 20ng/ml of TNFα for sixteen hours, after which they were harvested. B) Similar experiments were carried with the addition of 0.5ug of wild type APC. C) Similar experiments were repeated in HEK293 cells. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$.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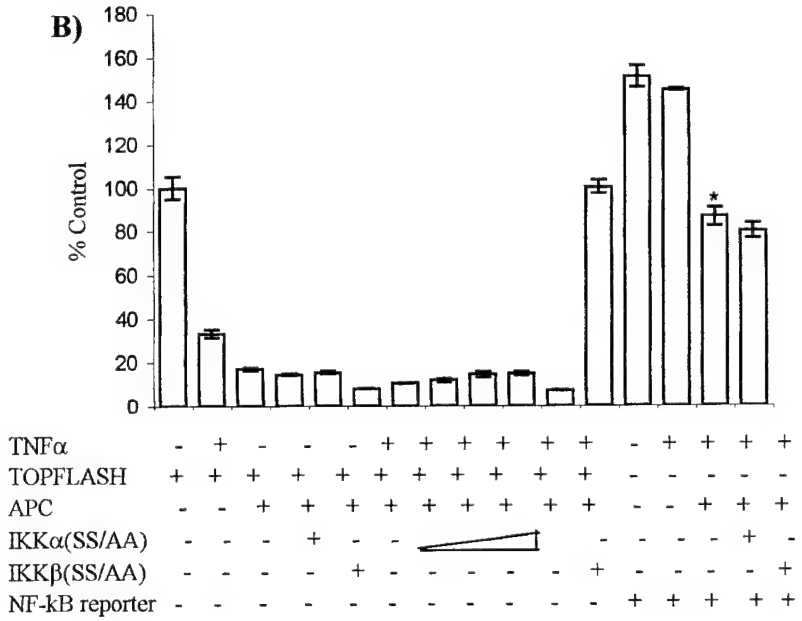
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C)

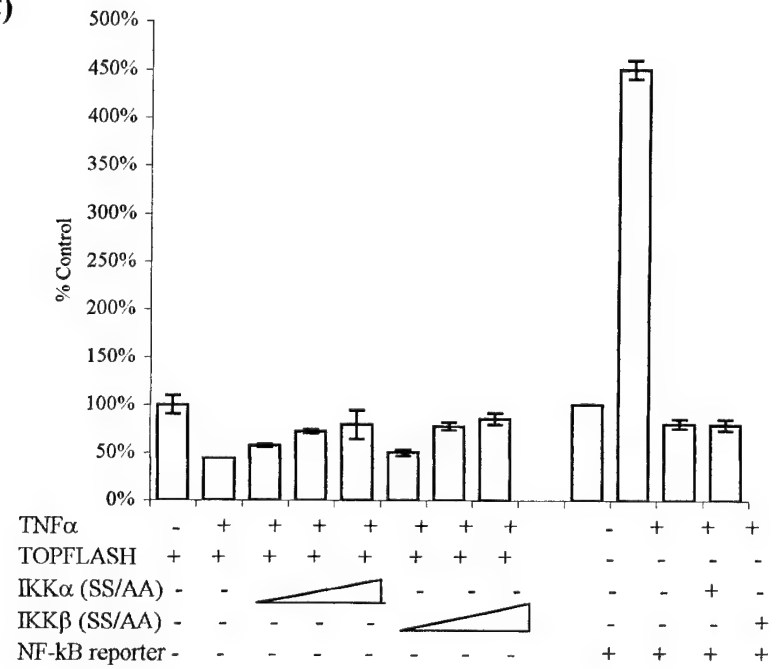
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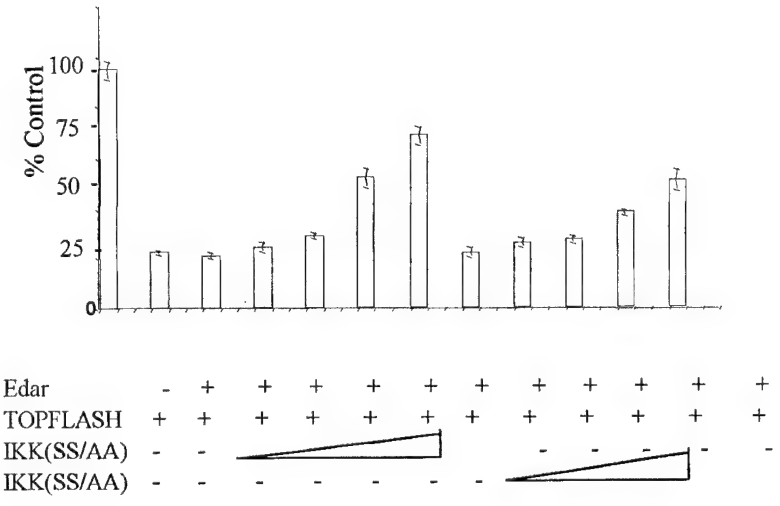
Figure 7: Effect of DN-IKK mutants on Eda/Edar regulation of β-catenin signaling activity. A) SW480 cells were transfected with 50ng of ectodysplasin receptor, Edar 0.1ug of Topflash or NFκB reporters together with DN-IKKα and DN-IKKβ (0.05-1.0ug) for twenty-four hours. B) Similar experiments with 0.1ug of -163cyclin D1Luc promoter were repeated. A cyclin D1 promoter with mutated TCF sites was used as negative controls. C) 50ng of Eda, 0.1ug of Topflash reporter and either 0.5 ug or 1.0ug of DN-IKK mutants were transfected into SW480 cells. D) Experiment was performed in Caco2 cells with 0.1ug, 0.5ug and 1.0 ug of DN-IKK mutants with 50ng of Edar. Similar amounts of reporters (0.1ug), Topflash, Fopflash and NFκB were used. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$.

A)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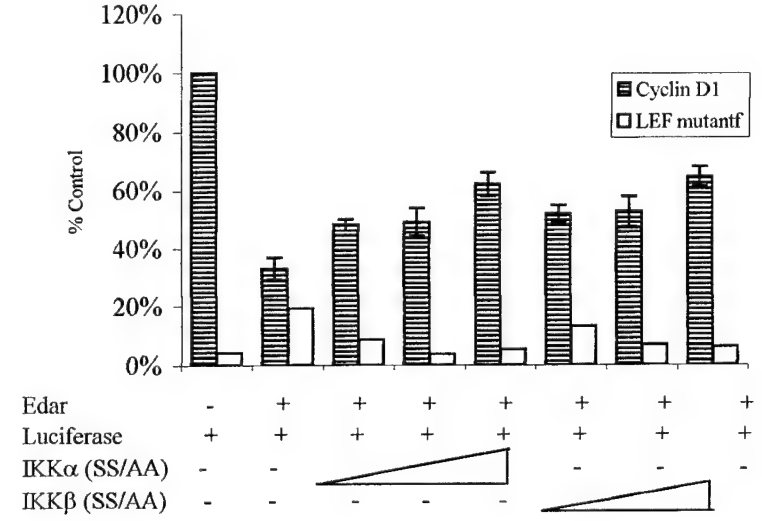
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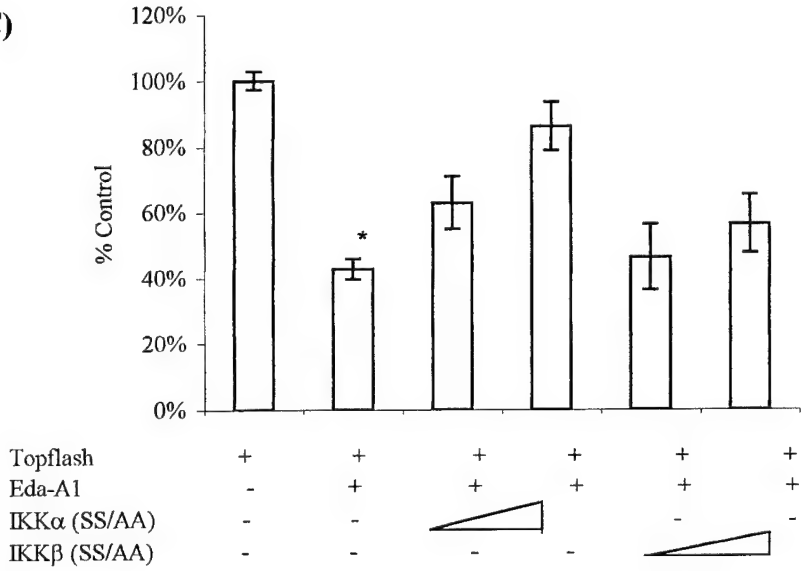
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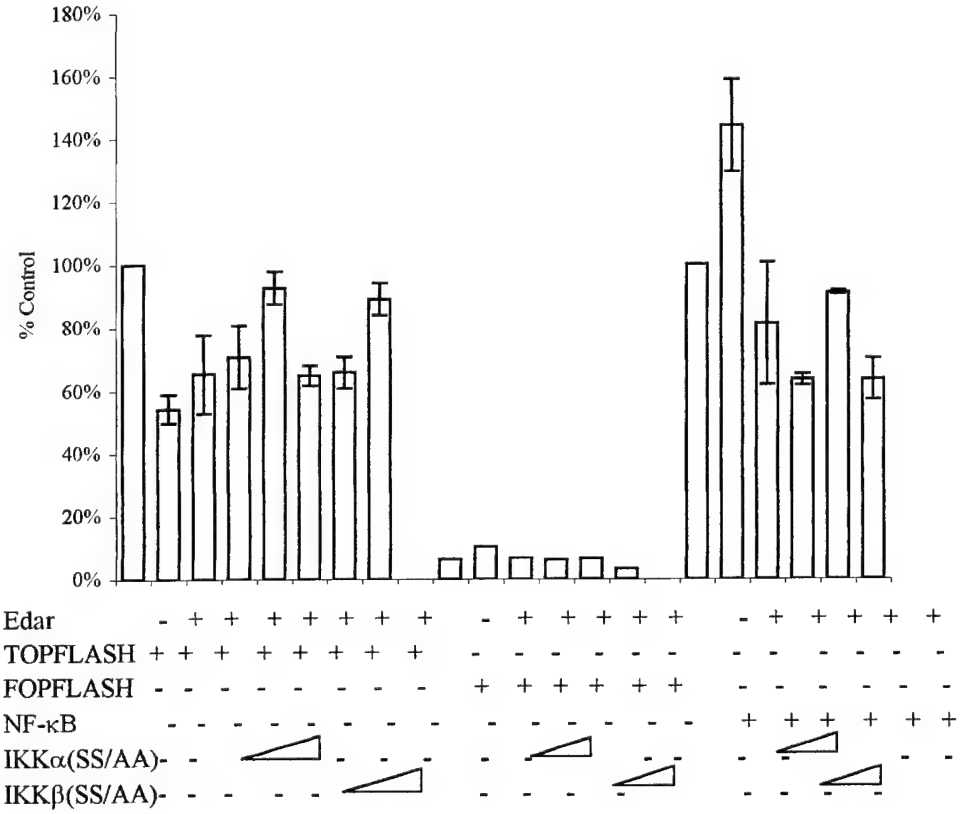
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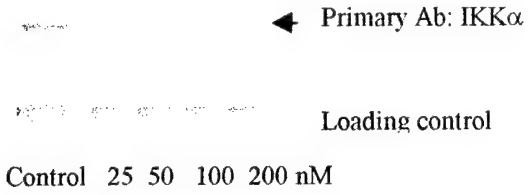
Figure 8. Effect of RNAi against IKKα and IKKβ on TNFα treated SW480 cells. A) SW480 cells were treated with increasing doses of RNAi against IKKα and IKKβ (from 25nM to 200nM) over seventy-two hours. Media was changed and cells were treated with 20ng/ml of TNFα for a further sixteen hours and whole cell lysates were made. (see in Materials and Methods). The top panel shows that the band corresponding to IKKα is decreased after treatment with 25nM of IKKα RNAi. B) Luciferase reporter assay. SW480 cells were treated with controls which were non-specific RNAi (NSRNAi) or 25nM of RNAi targeted against IKKα over seventy-two hours and transiently transfected with 0.1ug Topflash or NFκB reporter for twenty-four hours. C) As B but using 100nM of RNA targeted against IKKβ. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$.

A)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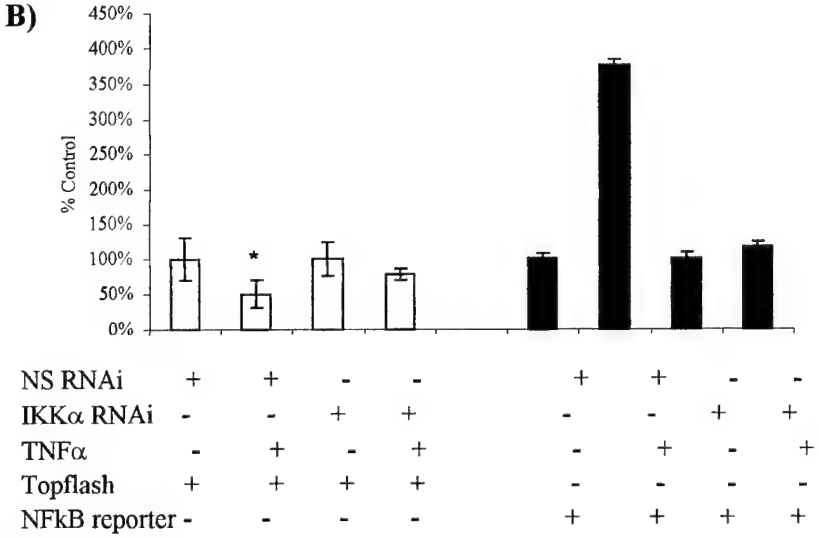
Loading control
Control 25 50 100 200 nM

B)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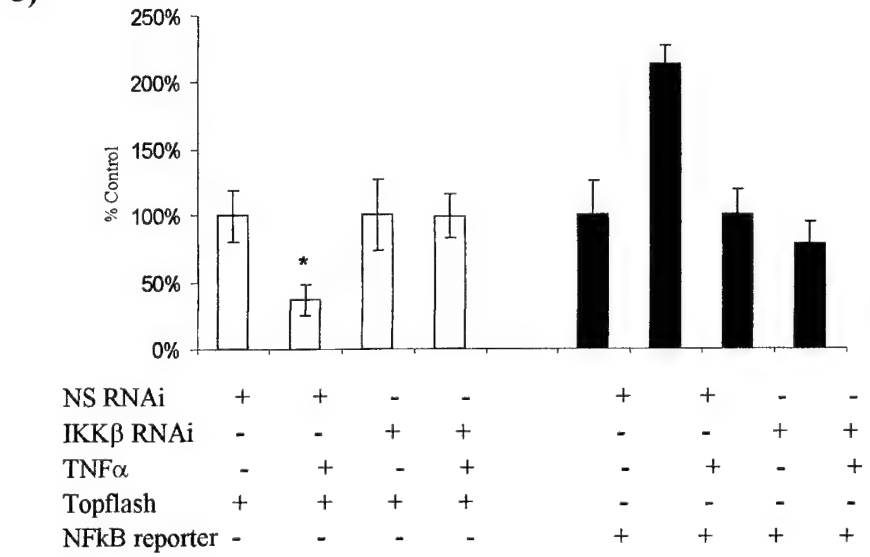
Topflash + + + + - - - -
NFkB reporter - - - - + + + +

C)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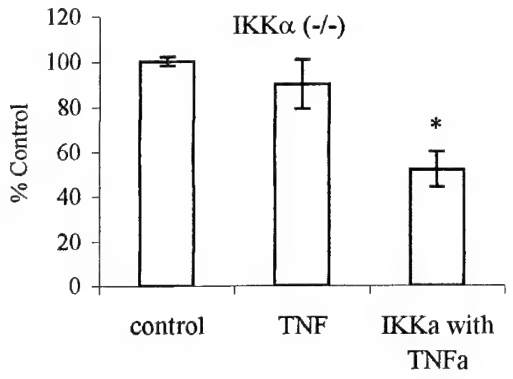
NFkB reporter - - - - + + + +

Figure 9: Effect of TNFα on β-catenin signaling in IKK (-/-) mouse embryonic fibroblast (MEF) cells. Wild Type MEF cells, IKKα (-/-) and IKKβ (-/-) cells were transfected with 0.1ug of Topflash or NFκB reporter for twenty four hours and treated with 20ng/ml of TNFα for a further sixteen hours. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$.

Figure 10: Effect of wild type (WT) IKKα and IKKβ on TNFα regulation of β-catenin signaling in MEF cells. A and D) IKKα (-/-) B and E) IKKβ (-/-) C and F) double knockouts IKKαβ (-/-) MEFs transfected with 0.1ug of Topflash reporter (A,B,C) or NFκB reporter (D,E,F) and 0.5ug of WT IKKs. After twenty-four hours, they were treated with 20ng/ml TNFα for sixteen hours. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$.

A)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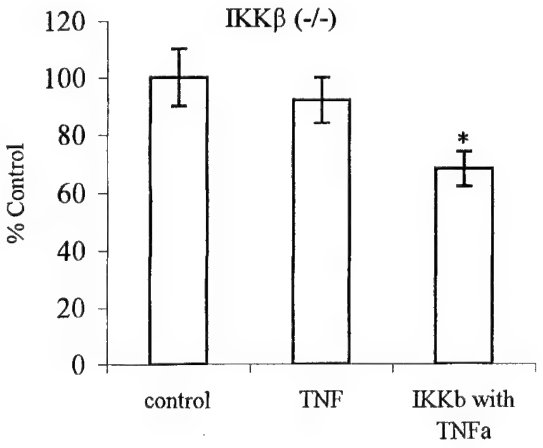
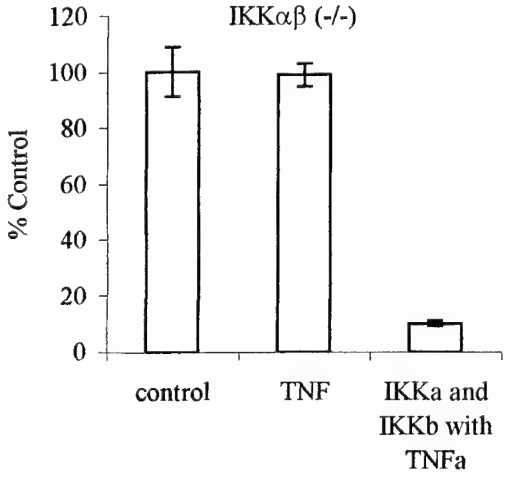
IKKa with TNFa

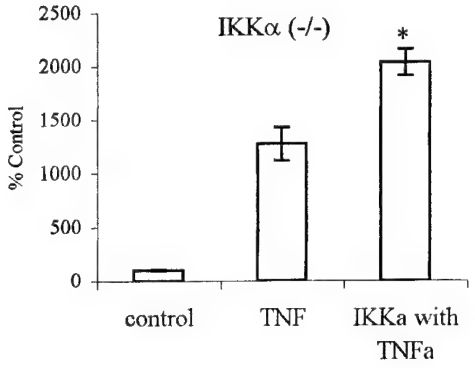
B)


IKKβ (-/-)
% Control
120
100
80
60
40
20
0
*
control
TNF
IKKb with TNFa

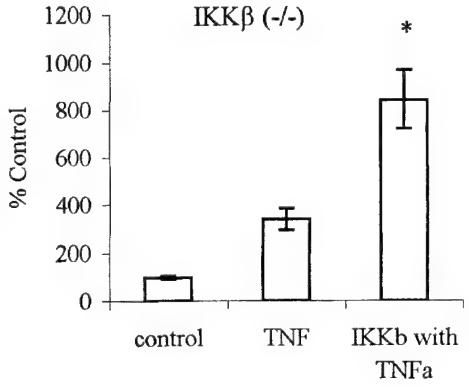
C)


IKKαβ (-/-)
% Control
120
100
80
60
40
20
0
control
TNF
IKKa and IKKb with TNFa

D)


IKKα (-/-)
2500
2000
1500
1000
500
0
% Control
*
control
TNF
IKKa with TNFa

E)


IKKβ (-/-)
1200
1000
800
600
400
200
0
% Control
*
control
TNF
IKKb with TNFa

F)

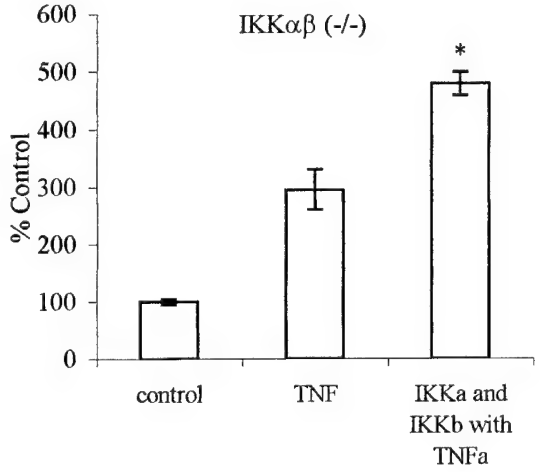
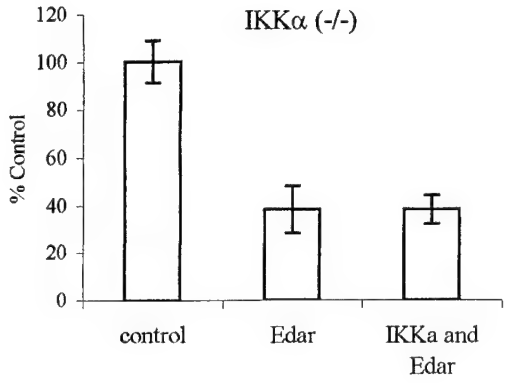
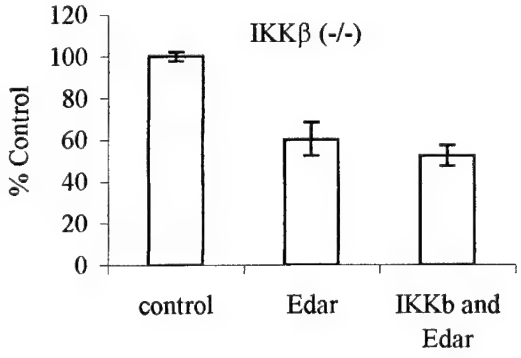

IKKαβ (-/-)
600
500
400
300
200
100
0
% Control
*
control
TNF
IKKa and IKKb with TNFa

Figure 11. Effect of WT IKK on Edar regulation of β-catenin signaling in IKK (-/-) MEF cells. A and D) IKKα (-/-) B and E) IKKβ (-/-) C and F) double knockouts IKKαβ (-/-) MEFs were transfected with 0.1ug Topflash reporter (A,B,C) or NFκB reporter (D,E,F), 50ng Edar and 0.5ug each of wild type IKKs for forty-eight hours. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test.* denotes statistical significance where $p < 0.05$.

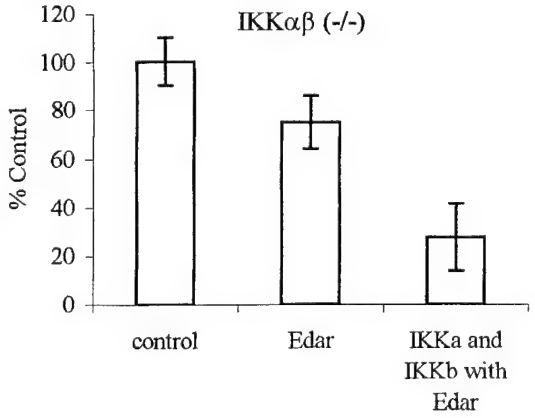
A)


IKKα (-/-)
% Control
120
100
80
60
40
20
0
control
Edar
IKKa and Edar

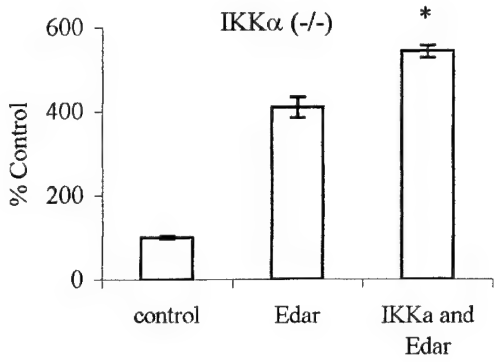
B)


IKKβ (-/-)
% Control
120
100
80
60
40
20
0
control
Edar
IKKb and Edar

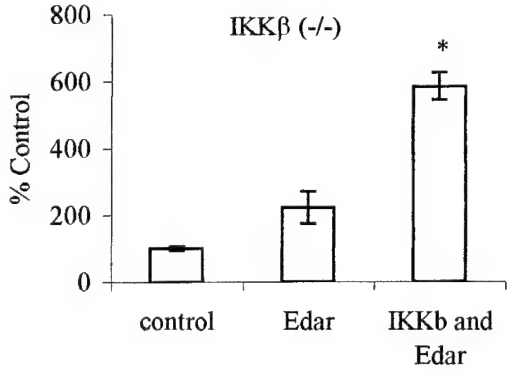
C)


IKKαβ (-/-)
% Control
120
100
80
60
40
20
0
control
Edar
IKKa and IKKb with Edar

D)


IKKα (-/-)
*
600
400
200
0
% Control
control
Edar
IKKa and Edar

E)


IKKβ (-/-)
*
800
600
400
200
0
% Control
control
Edar
IKKb and Edar

F)

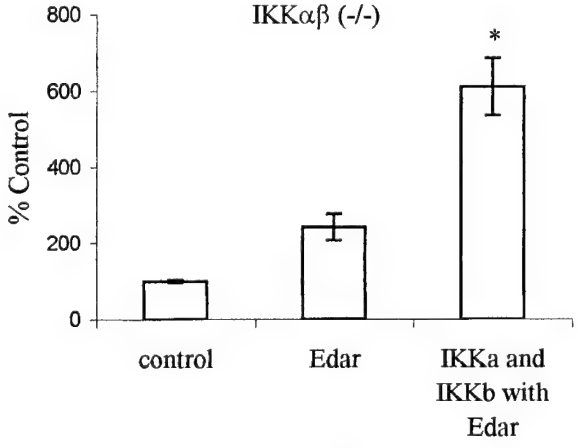

IKKαβ (-/-)
*
800
600
400
200
0
% Control
control
Edar
IKKa and IKKb with Edar

Figure 12: Effect of Edar on β-catenin signaling activity in IKK (-/-) MEF cells. Wild type and knockout MEF cells were transfected with 50ng of Edar together with 0.1ug of reporter for forty-eight hours. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$.

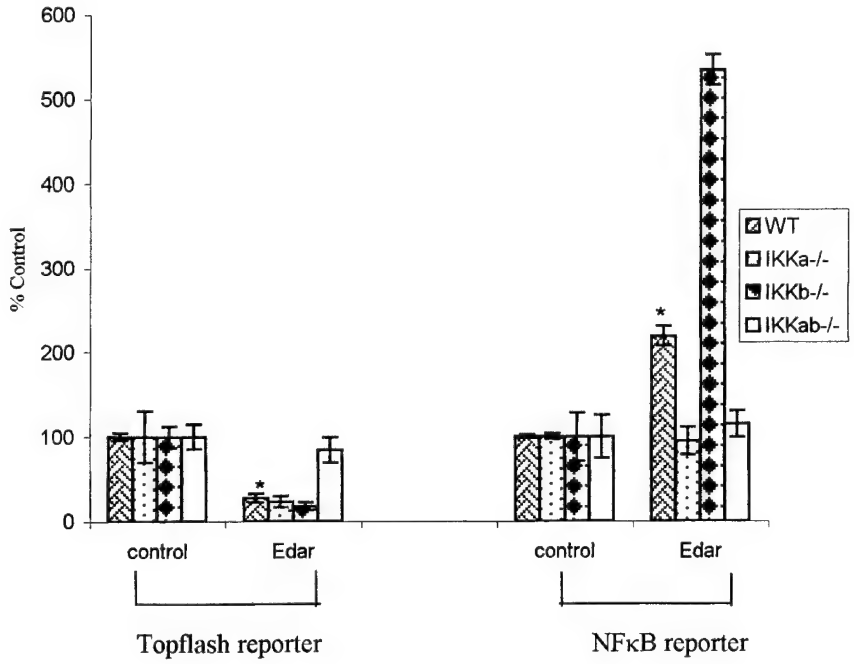
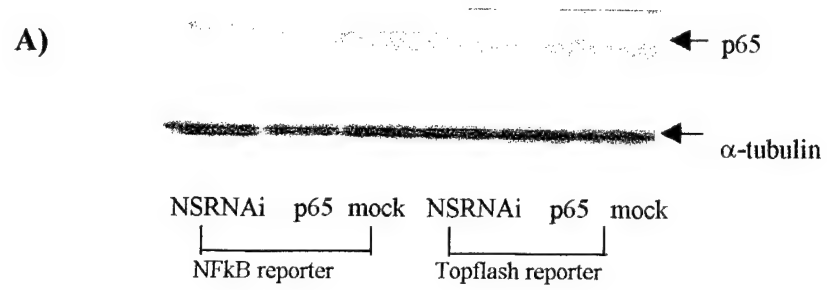
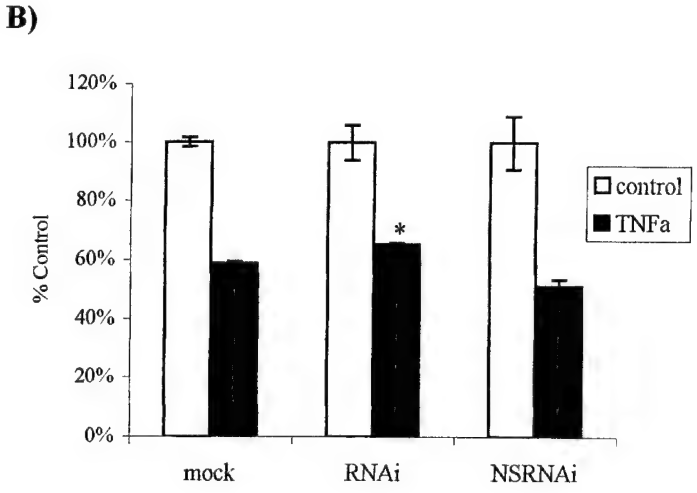
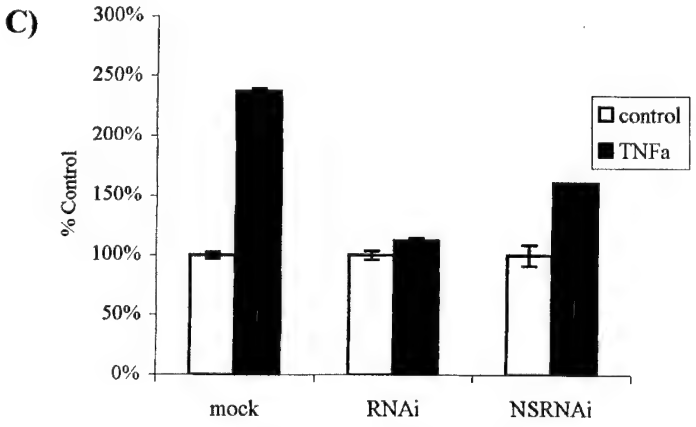

600
500
400
300
200
100
0
% Control
WT
IKKa-/-
IKKb-/-
IKKab-/-
control
Edar
control
Edar
Topflash reporter
NFκB reporter

Figure 13: Influence of NFκB activity on TNFα/Edar repression of β-catenin signaling. A) Western Blot. SW480 cells were treated with 80nM RNAi targeted against active NFκB, p65 subunit for seventy-two hours, harvested and lysed as described in Materials and Methods. Loading controls with α-tubulin are shown at the bottom panel. B) Topflash reporter assay. SW480 cells were treated with 80nM RNAi targeted against active NFκB, p65 subunit for seventy-two hours and transfected with 0.1ug of reporters, Topflash or NFκB for twenty four hours. 20ng/ml of TNFα was then added for sixteen hours. Cells were harvested and luciferase activity was measured. Mock transfections denote the use of transfecting reagent, Oligofectamine in the absence of RNAi and cells were treated the same way. Scrambled RNAi (NSRNAi) was also used as a control. The sequence is described in Materials and Methods. C) NFκB reporter assay. Cells were treated in the same way as described in the Topflash assay. D) SW380 cells were treated with non specific RNAi (NSRNAi) and RNAi against p65 subunit as B). However 50ng of Edar was transfected together with the reporters for twenty-four hours. In all the reporter assays, experiments were performed in triplicates and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$.


A)
p65
α-tubulin
NSRNAi p65 mock NSRNAi p65 mock
NFkB reporter
Topflash reporter


B)
120%
100%
80%
60%
40%
20%
0%
% Control
*
control
TNFa
mock
RNAi
NSRNAi


C)
300%
250%
200%
150%
100%
50%
0%
% Control
control
TNFa
mock
RNAi
NSRNAi

D)

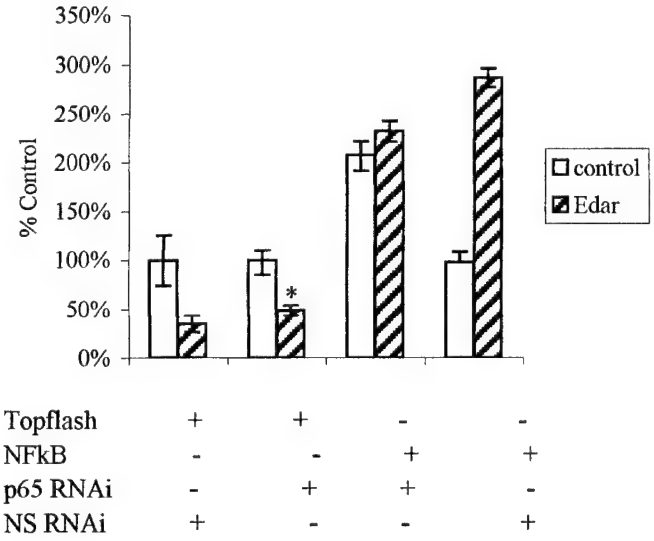
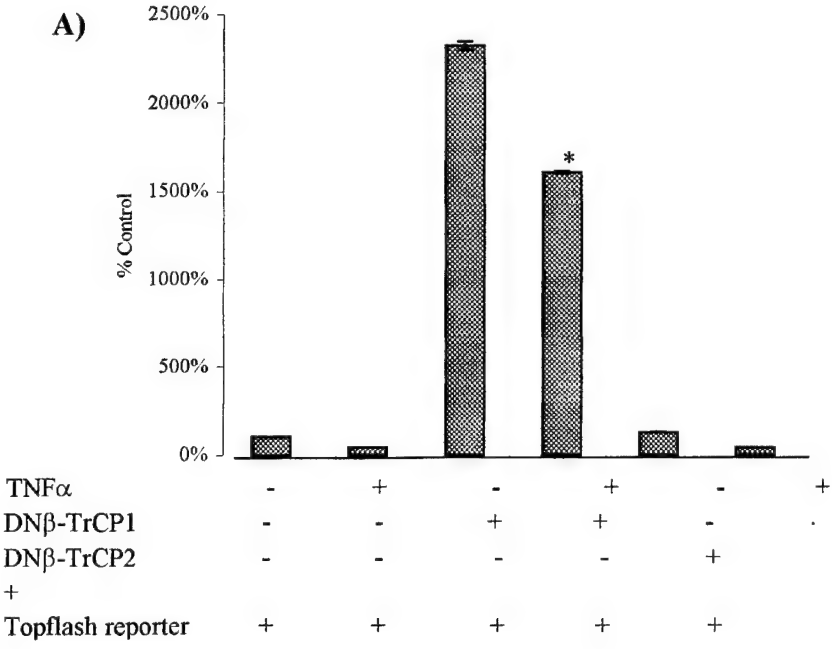

350%
300%
250%
200%
150%
100%
50%
0%
% Control
*
control
Edar
Topflash + + - -
NFkB - - + +
p65 RNAi - + + -
NS RNAi + - - +

Figure 14: Effect of dominant negative β-TrCP (DN β-TrCP) on TNFα regulation of β-catenin signaling activity. A) Topflash reporter assay in HEK293 cells. 0.5ug of DN β-TrCP1 and 0.1ug of Topflash reporter were transfected into 293 cells for twenty-four hours. After which, 20ng/ml of TNFα was added for sixteen hours. Cells were harvested and luciferase activity was measured. In all the reporter assays, experiments were performed in triplicates and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$. B) NFκB reporter assay. Experiment was repeated as in A) with 0.1ug of NFκB reporter transfected instead.


A)
2500%
2000%
1500%
1000%
500%
0%
% Control
*
TNFα - + - + - +
DNβ-TrCP1 - - + + - -
DNβ-TrCP2 - - - - + +
Topflash reporter + + + + + +

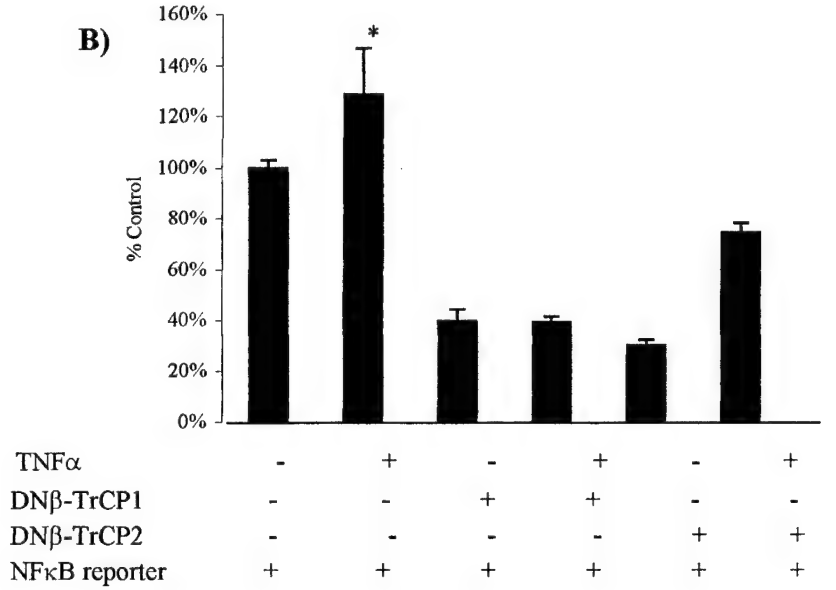
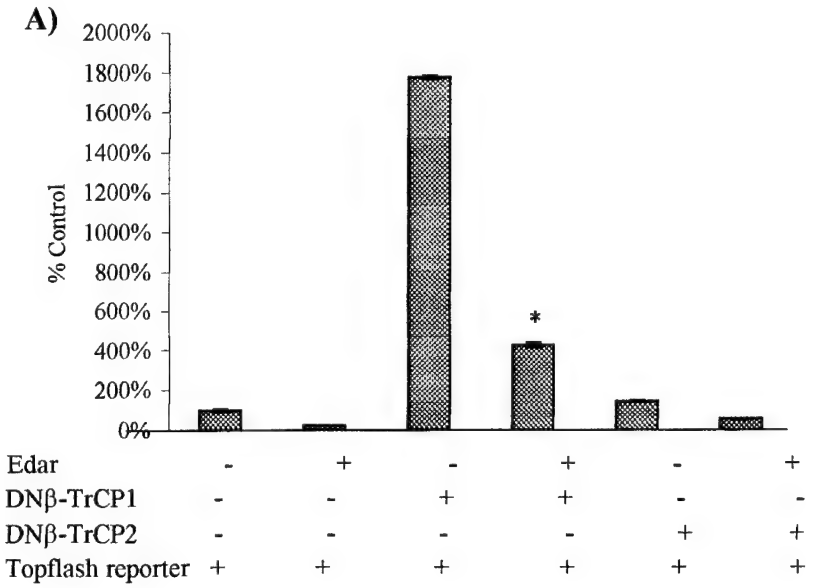

B)
160%
140%
120%
100%
80%
60%
40%
20%
0%
% Control
*
TNFα - + - + - +
DNβ-TrCP1 - - + + - -
DNβ-TrCP2 - - - - + +
NFκB reporter + + + + + +

Figure 15: Effect of dominant negative β-TrCP (DN β-TrCP) on Edar regulation of β-catenin signaling activity. A) Topflash reporter assay in HEK293 cells. 0.5ug of DN β-TrCP1, 0.1ug of Topflash reporter and 50ng of Edar were transfected into 293 cells for twenty-four hours. Cells were harvested and luciferase activity was measured. In all the reporter assays, experiments were performed in triplicates and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$. B) NFκB reporter assay. Experiment was repeated as in A) with 0.1ug of NFκB reporter transfected instead.


A)
2000%
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
% Control
*
Edar - + - + - +
DNβ-TrCP1 - - + + - -
DNβ-TrCP2 - - - - + +
Topflash reporter + + + + + +

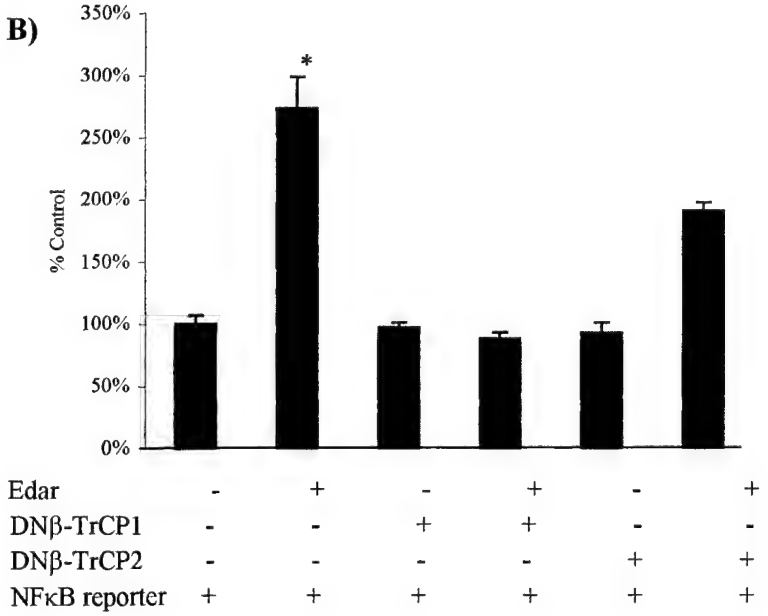
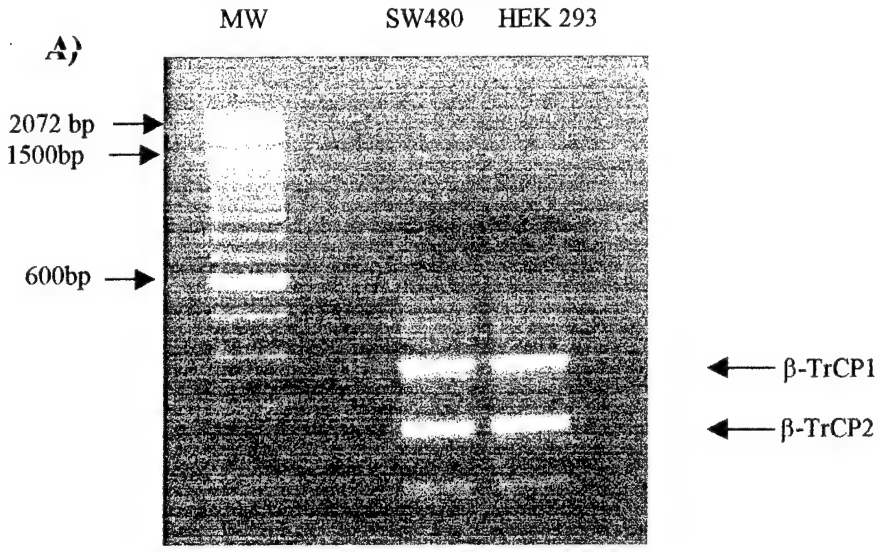

B)
350%
300%
250%
200%
150%
100%
50%
0%
% Control
*
Edar - + - + - +
DNβ-TrCP1 - - + + - -
DNβ-TrCP2 - - - - + +
NFκB reporter + + + + + +

Figure 16: Expression of β-TrCP in SW480 and HEK293 cells. A) RT-PCR of SW480 cells and HEK 293 cells. RNA were harvested from cells as described in Materials and Methods. 2ul of PCR product together with loading buffer, was loaded onto 1% agarose gel and allowed to run for one hour at 80V. There are 2 bands seen for each cell line, indicating that both F-box proteins, β-TrCP1 (364bp) and β-TrCP2 (256bp) are present. 100bp molecular weight marker (MW) is shown on extreme left. B) Schematic diagram of the location of the primers for each of the F-box protein. Similar primers were chosen for each protein. However, due to the absence of a 108 bp fragment in β-TrCP2, a size difference was observed.


MW
SW480
HEK 293
A)
2072 bp
1500bp
600bp
β-TrCP1
β-TrCP2

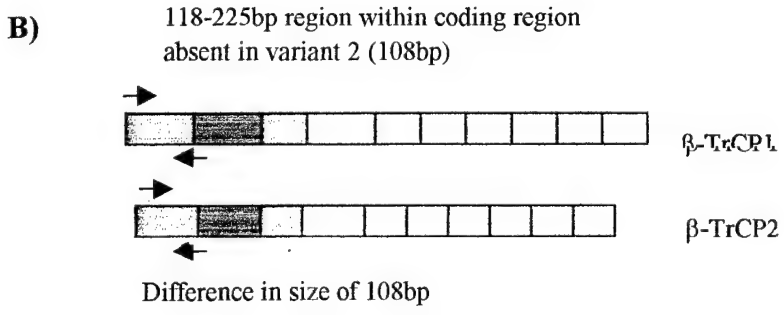
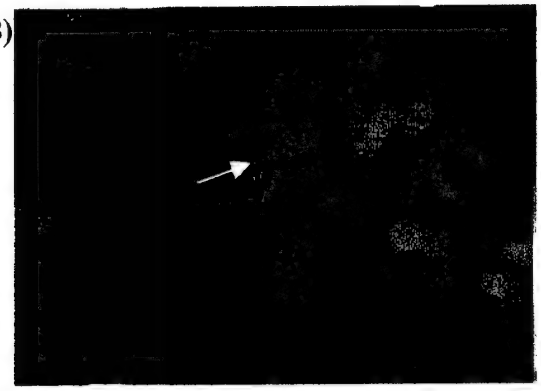

B)
118-225bp region within coding region
absent in variant 2 (108bp)
β-TrCP1
β-TrCP2
Difference in size of 108bp

Figure 17: Effect of constitutively active IKK (CA IKK) mutants on localization of total β-catenin. A) SW480 cells were transiently transfected with 1.0ug of flag-tagged CA IKKα for twenty-four hours. Cells were fixed and stained as described in Materials and Methods. Staining was performed first using polyclonal flag antibody and then fluorescein-conjugated secondary antibody (green) (transfected cells indicated by arrow). B) Cells were stained for total β-catenin using monoclonal C-terminus β-catenin antibody and then Texas Red-conjugated secondary antibody. (red) (same cell transfected with CA IKK mutant indicated by arrow) C) A superimposed image of both A) and B).

A)

B)



C)

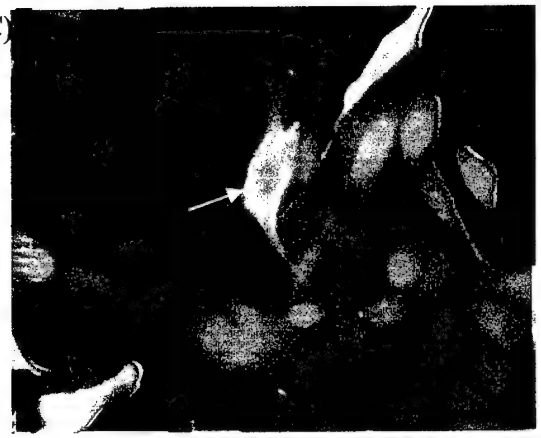
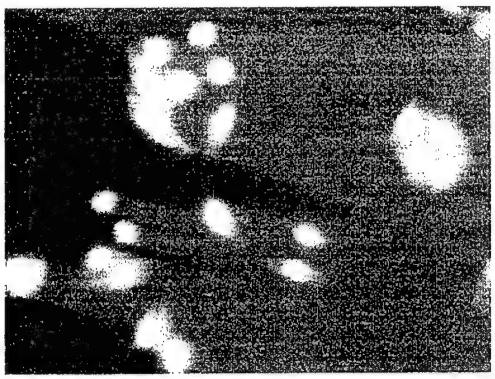
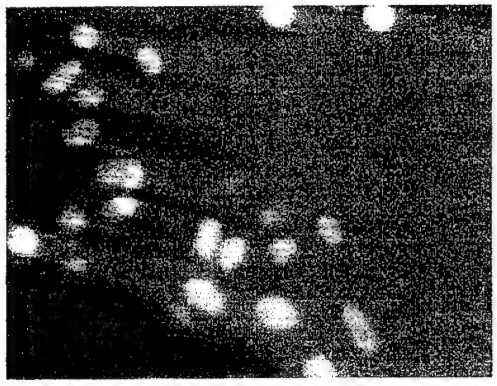


Figure 18: Effect of TNFα on localization of de-phosphorylated β-catenin in SW480 cells. A) Control SW480 cells without treatment with TNFα. B) SW480 cells were treated with 20ng/ml TNFα for sixteen hours and monoclonal αABC (de-phosphorylated β-catenin) antibody and fluorescein-conjugated secondary antibody were used. Staining was performed as described in Materials and Methods. Pictures were taken at similar exposure times (indicated by green staining) C) Statistical analysis of cells with no nuclear staining before and after treatment with TNFα. 7 fields of view at 200X magnification were randomly selected and the number of cells were counted and the average calculated. Statistical analysis was performed using paired Student's T-test. * indicates statistical significance between treatments, where $p < 0.05$.

A)



B)



C)

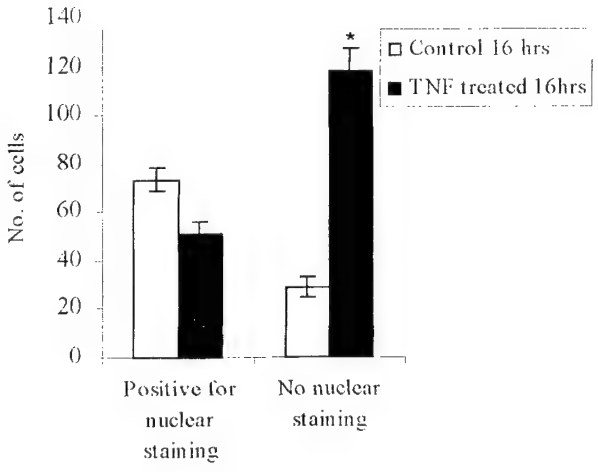
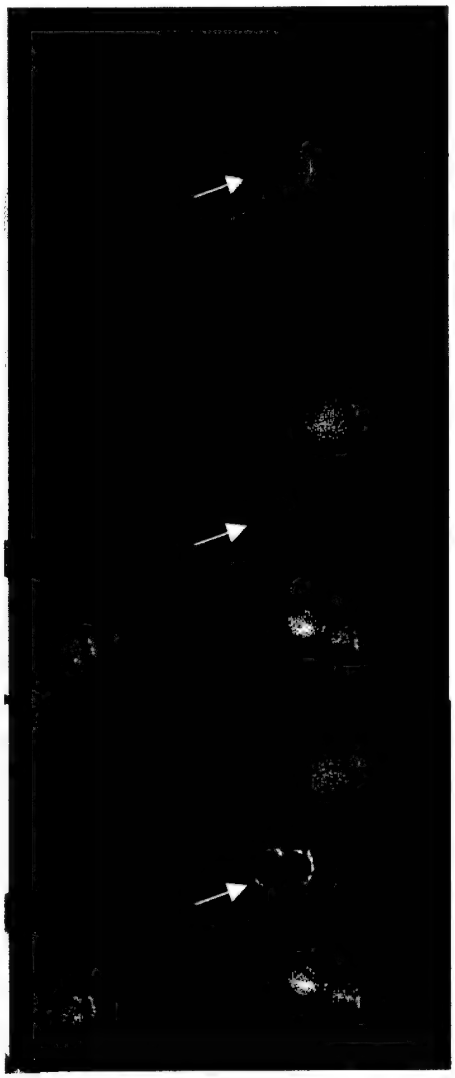

140
120
100
80
60
40
20
0
No. of cells
*
Control 16 hrs
TNF treated 16hrs
Positive for nuclear staining
No nuclear staining

Figure 19: Effect of overexpression of Edar on localization of de-phosphorylated β-catenin protein in SW480 cells. SW480 cells were transfected with 50ng of flag-tagged Edar for twenty-four hours. Cells were fixed and stained as described in Materials and Methods. A) SW480 cells stained with polyclonal anti-flag antibody and Texas red-conjugated secondary antibody (cells transfected with Edar are stained red and denoted by arrow) B) Same field of view showing cells stained with fluorescein-conjugated monoclonal αABC (cells are stained green). C) Superimposed image of A) and B). Same arrow denotes cells that are transfected with Edar do not have any nuclear staining. D) Statistical analysis of the number of cells with nuclear staining. Average cell counts of 7 field of views at 200X magnification for control (cells not transfected) and cells transfected with Edar. Statistical analysis was performed using paired Student's T-test. * indicates statistically significant, where $p < 0.05$.

A)

B)

C)



D)

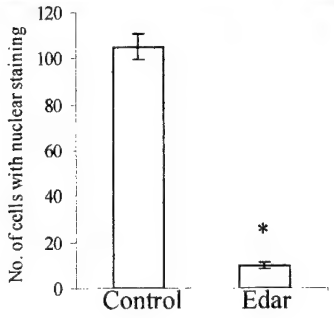
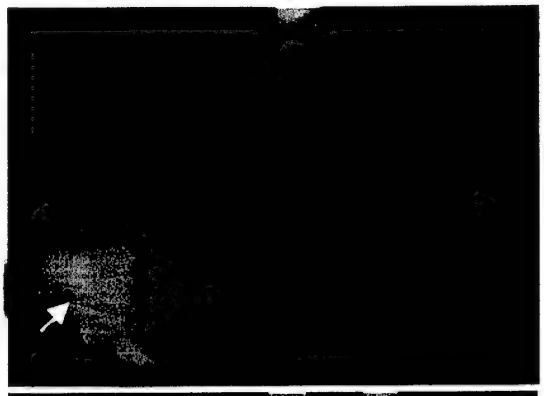

No. of cells with nuclear staining
120
100
80
60
40
20
0
*
Control
Edar

Figure 20: Effect of over-expression of constitutively active IKK (CA IKK) mutants on localization of de-phosphorylated β-catenin in SW480 cells. SW480 cells were transfected with 1.0ug of CA IKKα for twenty-four hours. Cells were fixed and stained as described in Materials and Methods. A) Cells were first probed with polyclonal flag antibody and then stained using Texas Red-conjugated secondary antibody (red). B) Cells were stained using antibody directed against β-catenin de-phosphorylated at Ser37, Thr41 (green). Cells transfected with IKK mutant did not have any nuclear staining of β-catenin (indicated by arrow) when compared to control (cells without IKK). C) superimposed image of A) and B) D)-E) Statistical analysis of the number of cells with nuclear staining. Average cell counts of 7 field of views at 200X magnification for CA IKKα (D) and IKKβ (E). Statistical analysis was performed using paired Student's T-test. * indicates statistical significance, where $p < 0.05$.

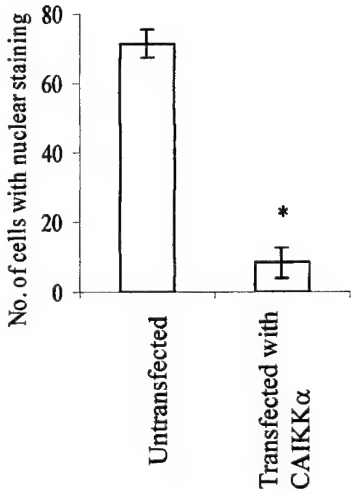
A)

B)



C)

D)


No. of cells with nuclear staining
80
60
40
20
0
*
Untransfected
Transfected with CAIKKα

E)

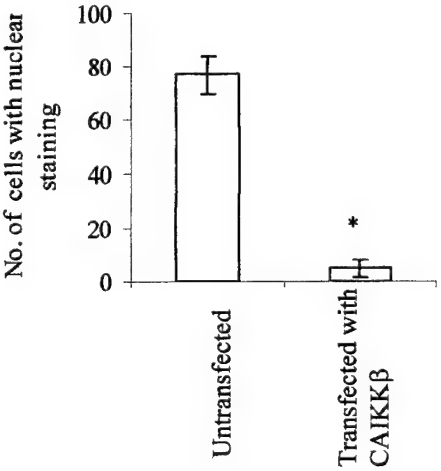
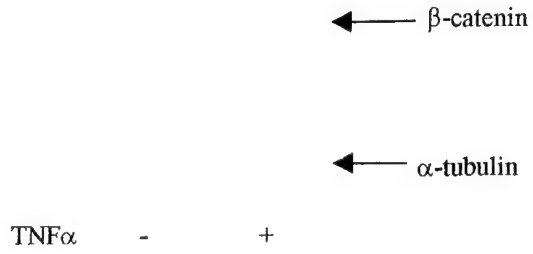

No. of cells with nuclear staining
100
80
60
40
20
0
*
Untransfected
Transfected with CAIKKβ

Figure 21: Effects of TNFα on total β-catenin protein in SW480 cells. A) Western analysis of cytoplasmic fraction of SW480 cells after treatment with 20ng/ml of TNFα for sixteen hours. Analysis was performed as described in Materials and Methods. Experiments were repeated at least three times. B) Cytoplasmic fraction of HEK293 cells after treatment with 20ng/ml of TNFα for similar period of time. β-catenin antibody that detects the C-terminus was used. Loading controls were done using α-tubulin and is shown at the bottom of each panel.

A)



B)

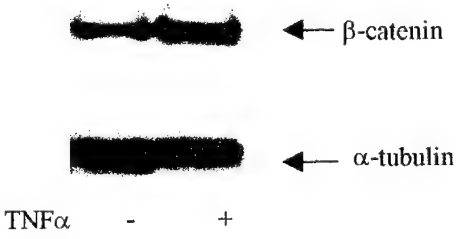


Figure 22: Effects of TNFα treatment on levels of phosphorylated β-catenin over time in SW480 cells. Western analysis of the cytoplasmic fraction of SW480 cells after treatment with 20ng/ml of TNFα over a time course of six hours. Cells were harvested at one minute, thirty minutes, two hours and six hours after treatment with TNFα. Analysis was performed as described in Materials and Methods. Experiments were repeated at least three times. Bands were compared to its own control at each time point. A polyclonal β-catenin antibody that detects β-catenin phosphorylated at Ser33,37,Thr41 was used. Antibodies to detect β-catenin de-phosphorylated at Ser37,Thr41 and total β-catenin were monoclonal antibodies. Bottom panel indicate loading control with α-tubulin.

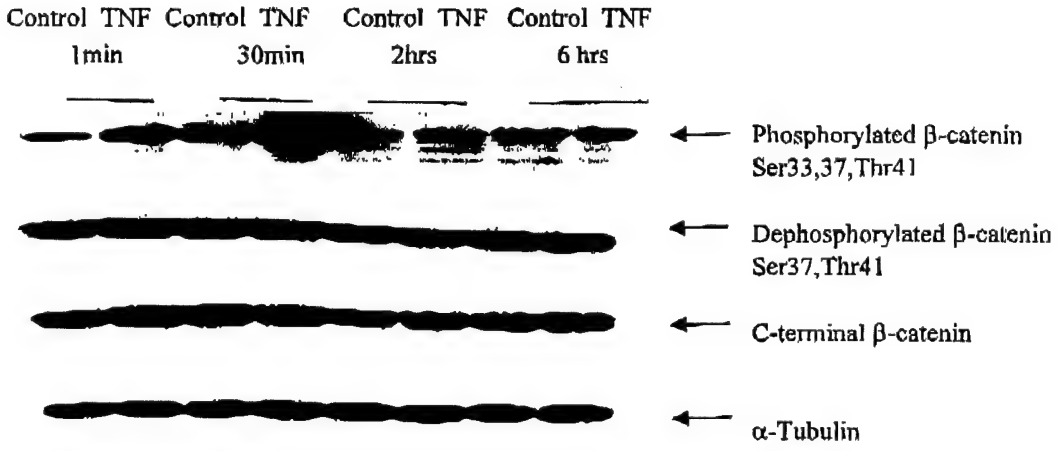
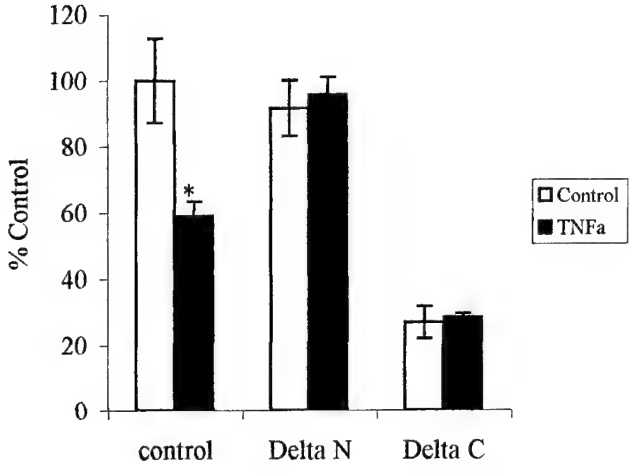

Control TNF Control TNF Control TNF Control TNF
1min 30min 2hrs 6 hrs
Phosphorylated β-catenin
Ser33,37,Thr41
Dephosphorylated β-catenin
Ser37,Thr41
C-terminal β-catenin
α-Tubulin

Figure 23: Effect of TNFα on truncated forms of β-catenin. A) Wild type MEF cells were transfected with 0.1ug of Topflash reporter and 0.5ug of either wild type β-catenin or deletion mutants of β-catenin for twenty-four hours. Cells were treated with 20ng/ml TNFα for an additional sixteen hours and luciferase activity was measured. Mutant forms of β-catenin, delta N (truncated N-terminus), delta C (truncated C-terminus) were made via PCR and their primers are described in Materials and Methods. B) Similar experiments were carried out with over-expression of 0.1ug of Topflash reporter, 50ng of Edar and 0.5ug of different forms of β-catenin. Cells were incubated for twenty-four hours. In all the reporter assays, experiments were performed in triplicates and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$.

A)


120
100
80
60
40
20
0
% Control
*
control
Delta N
Delta C
Control
TNFa

B)

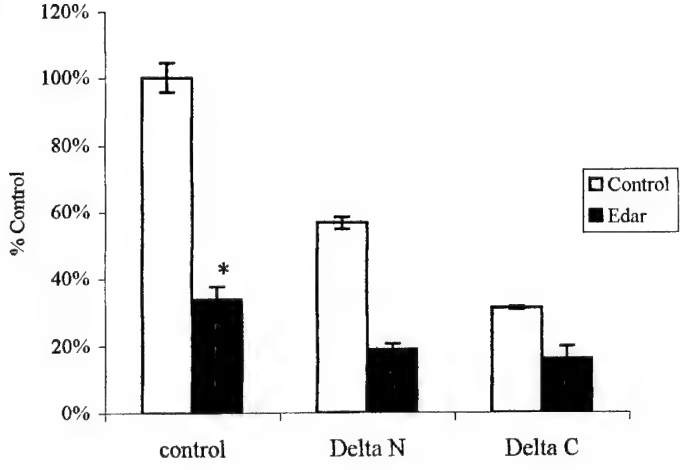
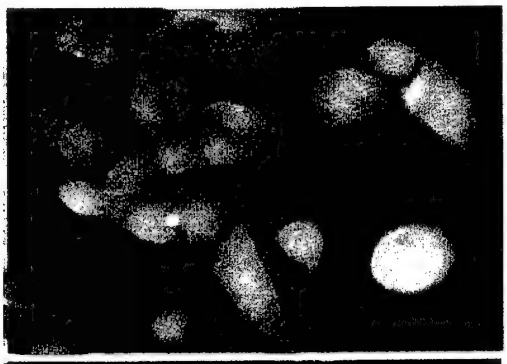

120%
100%
80%
60%
40%
20%
0%
% Control
*
control
Delta N
Delta C
Control
Edar

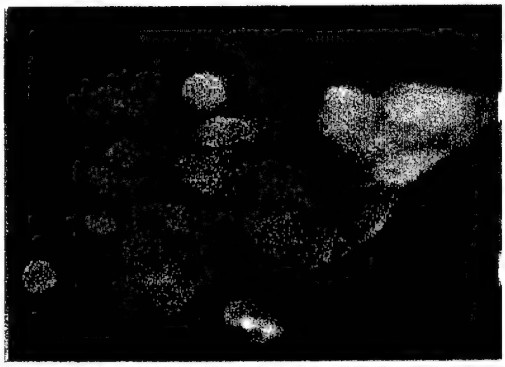
Figure 24: Localization of phosphorylated forms of β-catenin in SW480 cells after TNFα treatment. SW480 cells were treated with 20ng/ml of TNFα for 30 mins. Cells were fixed and stained as described in Materials and Methods. A) Cells without treatment (controls) were stained with antibody that recognizes β-catenin phosphorylated at Thr41/Ser45. B) Similar staining was performed on cells treated with TNFα. C) Cells without treatment (controls) were stained with antibody that recognizes β-catenin phosphorylated at Ser33, Ser37, Thr41. D) Similar staining was done with cells treated with TNFα. Experiments were repeated three times.

A)



B)

C)



D)

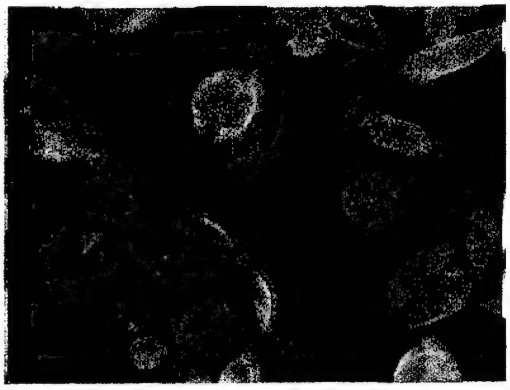
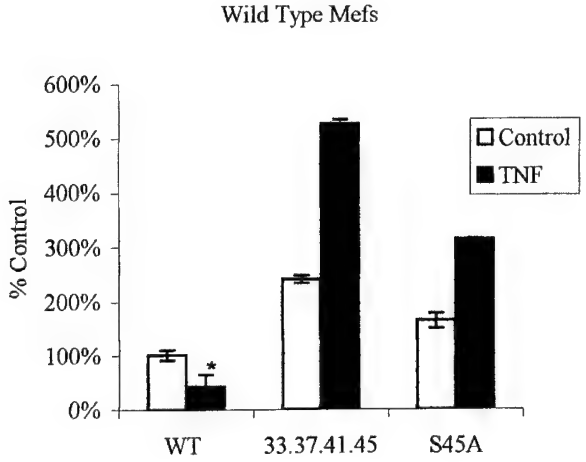
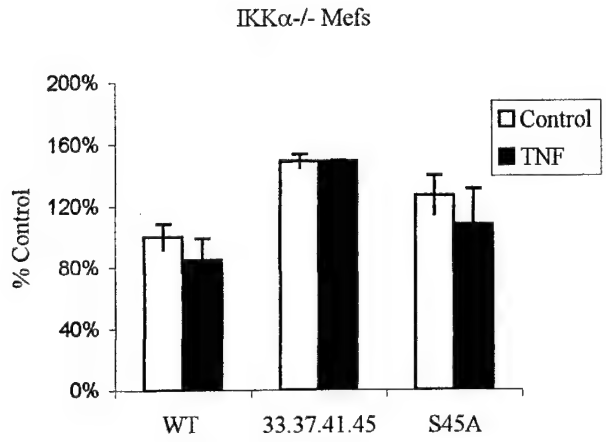


Figure 25: Effect of point mutants of β-catenin on TNFα repression of β-catenin signaling activity. A) Wild type mouse embryonic fibroblast cells (MEFs) were transfected with 0.5ug of wild type β-catenin, β-catenin mutated on residues 33,37,41,45 or β-catenin mutated on 45 alone for twenty-four hours. Cells were then treated with 20ng/ml of TNFα for further sixteen hours and harvested. Luciferase activity was measured. In all the reporter assays, experiments were performed in triplicates and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$. B) Similar experiments were performed in IKKα (-/-) cells. C) Similar experiments were performed in IKKβ (-/-) cells.

A)


Wild Type Mefs
600%
500%
400%
300%
200%
100%
0%
% Control
Control
TNF
*
WT
33.37.41.45
S45A

B)


IKKα-/- Mefs
200%
160%
120%
80%
40%
0%
% Control
Control
TNF
WT
33.37.41.45
S45A

C)

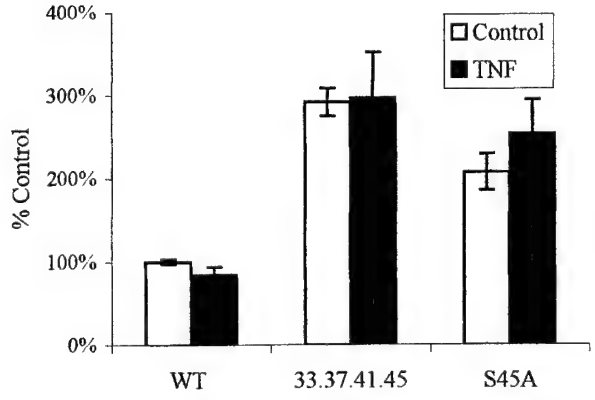

IKKβ-/- Mefs
400%
300%
200%
100%
0%
% Control
Control
TNF
WT
33.37.41.45
S45A

Figure 26: Effect of CA IKK mutants on β-catenin signaling activity. HCT 116 wt+/mut+ parental cells were transfected with 0.5ug of CA IKKα or IKKβ, 0.1ug of Topflash reporter for twenty-four hours. Cells were harvested and luciferase activity was measured. Luciferase activity was measured. In all the reporter assays, experiments were performed in triplicates and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$.

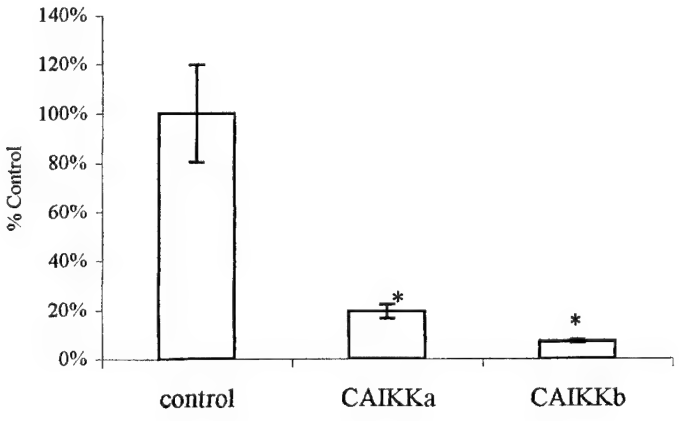

140%
120%
100%
80%
60%
40%
20%
0%
% Control
*
*
control
CAIKKa
CAIKKb

Figure 27: Effect of TNFα on β-catenin signaling in different forms of HCT-116 cell lines. Three different cell lines were used. WT+/Mut+ parental cells, and where either wild type (WT-/Mut+) (Mut) or mutant Ser45 allele (WT+/Mut-) (WT) was removed. These cell lines were transfected with 0.1ug of Topflash reporter and treated with 20ng/ml of TNFα over sixteen hours. Cells were harvested and luciferase activity was measured. In these reporter assays, experiments were performed in triplicates and repeated three times. Results were plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$.

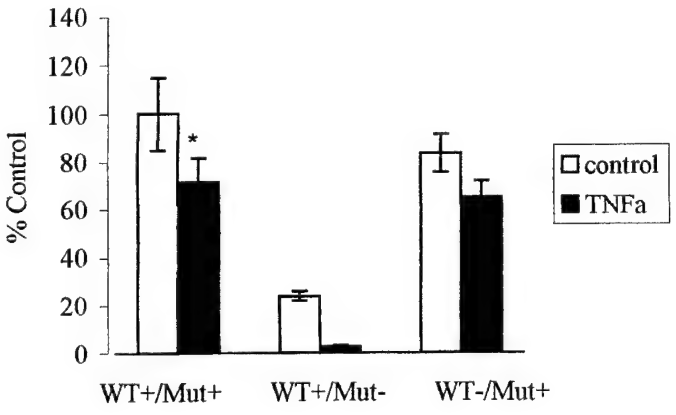

140
120
100
80
60
40
20
0
% Control
*
control
TNFa
WT+/Mut+
WT+/Mut-
WT-/Mut+

Figure 28: Effect of mutated β-catenin on Edar regulation of β-catenin signaling in wild type MEF cell lines. Wild Type mouse embryonic fibroblast cells (MEFs) were transfected with 0.5ug of wild type β-catenin, or mutated β-catenin at Ser45, Thr41, Ser45 and Ser33,Ser37,Thr41,Ser45, 50ng Edar and 0.1ug of Topflash reporter for twenty-four hours. In these reporter assays, experiments were performed in triplicates and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$.

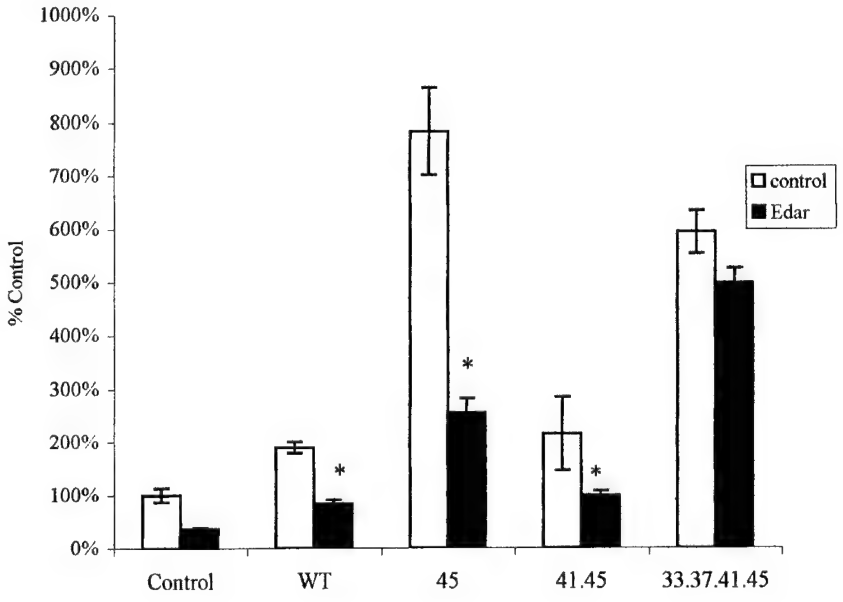
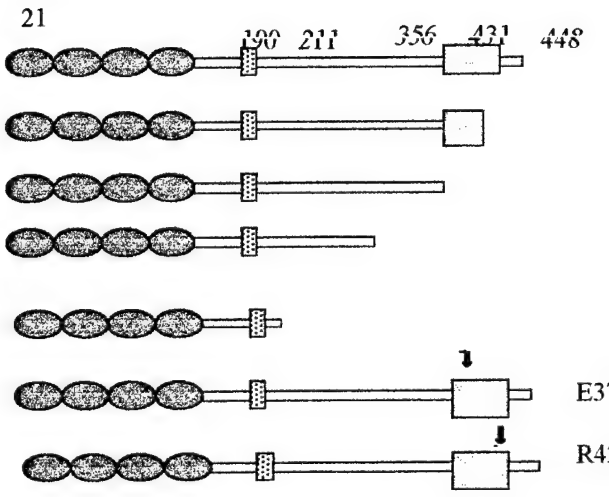
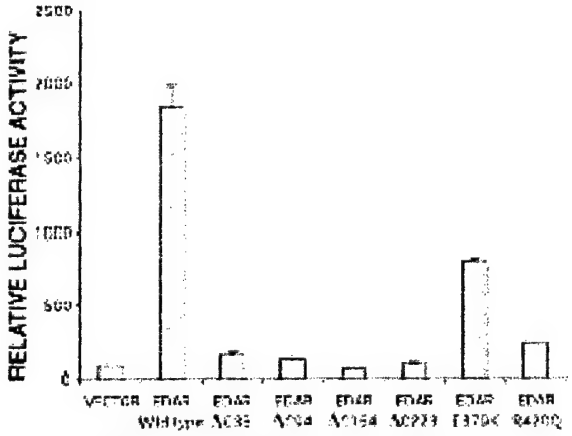

1000%
900%
800%
700%
600%
500%
400%
300%
200%
100%
0%
% Control
control
Edar
*
*
*
Control
WT
45
41.45
33.37.41.45

Figure 29: Involvement of the intracellular death domain of Edar in β-catenin signaling activity. A) Schematic representation of wild type Edar and its mutants. B) NFκB reporter assay reproduced from paper published by Chaudhary et al. (JBC, 276;2668-2677, 2001) HEK 293 cells were transfected with 0.5ug of wild type Edar or its mutants, along with an NFκB luciferase reporter construct (75 ng/well) and a Rous sarcoma virus promoter-driven β-galactosidase reporter construct (pRcRSV/LacZ; 75 ng) for twenty-four hours. Results were repeated three times and luciferase values were normalized with β-galactosidase activity to control for the difference in the transfection efficiency. C) Luciferase assay. SW480 cells were transfected with of 50ng of wild type Edar or its mutants and 0.1ug of Topflash reporter for twenty-four hours. In these reporter assays, experiments were performed in triplicate and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. * denotes statistical significance where $p < 0.05$.

A)

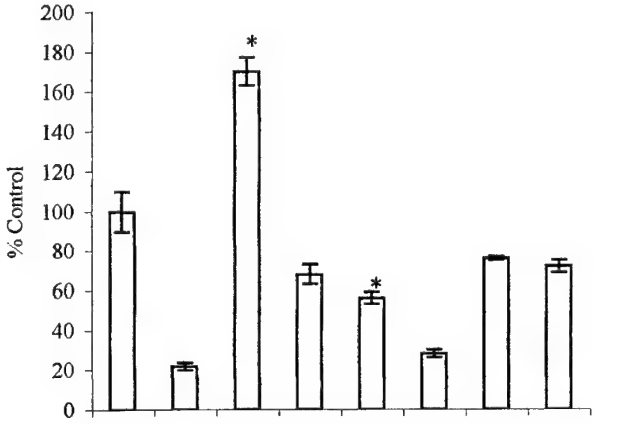

21
190
211
356
431
448
Wild Type Edar
C38
C94
C164
C223
E379K (autosomal recessive)
R420Q (autosomal dominant)

B)



(JBC, 276;2668-2677, 2001)

C)



Control	+	-	-	-	-	-	-	-
WT	-	+	-	-	-	-	-	-
C223	-	-	+	-	-	-	-	-
C38	-	-	-	+	-	-	-	-
C164	-	-	-	-	+	-	-	-
E379K	-	-	-	-	-	+	-	-
R420Q	-	-	-	-	-	-	+	-
C94	-	-	-	-	-	-	-	+

Key research Accomplishments:

1. TNFα and ectodysplasin receptor, Edar regulate β-catenin signaling activity
2. β-catenin and cyclin D1 are localized in the nuclei of epidermal cells in IKKα (-/-) mouse
3. Constitutively Active IKKα and β further Down-regulate β-catenin Signaling Activity and Augment NFκB Signaling Activity
4. Dominant Negative IKKα and β Reverse TNFα and Edar down-regulation of β-catenin Signaling Activity and Block NFκB Signaling Activity
5. TNFα Repression of β-catenin Signaling Activity is Abolished by IKK RNAi and in IKK (-/-) mouse embryonic fibroblast cells
6. NFκB signaling activity is not directly involved in TNFα/Edar repression of β-catenin Signaling
7. β-TrCP is not involved in down-regulating β-catenin Signaling
8. TNFα/ Edar decreases nuclear de-phosphorylated β-catenin
9. TNFα/ Edar do not change total β-catenin protein levels
10. TNFα repression of β-catenin activity involves phosphorylation of N-terminal serine and threonine residues

Reportable Outcomes:

1 manuscript for submission
PhD degree to be awarded Spring 2004

Conclusion:

Initial data using DN IKK mutants show that TNFα repression of β-catenin is blocked when either IKKα or IKKβ activity is blocked. In the case of ectodysplasin/ β-catenin signaling pathway, similar results were observed. However, TNFα and Edar behaved differently when IKK knockout MEF cells were used. Whereas TNFα mediated repression of β-catenin signaling was blocked in the absence of either form of IKK, both IKKα and IKKβ needed to be deleted to block Edar's effects on β-catenin signaling. Over-expressing DN IKKs results in the expression of large amounts of exogenous DNA that overwhelms and distrupts the endogenous IKK complex to prevent it from phosphorylating or

activating downstream proteins. Knockout studies are advantageous because the gene of interest is removed. Over-expression of mutant plasmids quantitatively interfere with signal transduction molecules while gene knockouts qualitatively eliminate signal transduction molecules. The most likely explanation for the differences in the role of the IKK complex in TNFα and Edar pathways is as follows. It is possible that TNFα is able to utilize IKK heterodimers but not homodimers and Edar is only able to transduce signals through IKKα and IKKβ homodimers. This may explain why either knocking out IKKα or IKKβ prevents TNFα from repressing β-catenin signaling activity in MEF cells. When Edar is over-expressed, presence of either IKKα or IKKβ in single knockouts allow homodimers to form and β-catenin is still able to be repressed. There is some debate in the IKK field over the relative role of IKK hetero-and homodimers[132;134;135;206;207;210;239]. There are differing opinions on whether IKKα and IKKβ serve different functions within the cell and it is possible that the removal of both kinases effectively removes a number of reserve pathways that result in repression of β-catenin signaling activity. It should also be pointed out that the IKK complex consists of IKKα, IKKβ and IKKγ. It would be interesting to repeat experiments using IKKγ (-/-) cells since they have a phenotype similar to IKKβ (-/-) and cells are expected to be insensitive to the effects of TNFα and Edar. This only highlights the complication of working out the detail of the signaling pathway within the cell. Further work needs to be done to elucidate the upstream mediators involved in the ectodysplasin pathway in order to understand the role that it plays in epithelial development and how it regulates β-catenin signaling.

Our results indicate a novel pathway for cytokines to regulate β-catenin signaling without an increase in degradation of β-catenin protein via the known F-box protein, β-TrCP. It is possible that degradation may occur but through other F-box proteins. If it is involved, addition of TNFα or Edar would not be able to reduce β-catenin signaling. On the other hand, experiments with β-catenin mutants of the N-terminal phosphorylation sites seem to indicate otherwise. These phosphorylation sites that target β-catenin for proteosomal degradation, including Ser33, Ser37, Thr 41 and Ser45 appear to have another role, which does not involve β-TrCP. Instead, TNFα and Edar are

able to regulate the phosphorylation and subsequently the signaling activity of β-catenin, independently of degradation. Reporter assays show that Ser45 plays an important role in TNFα regulation of β-catenin signaling activity since a single point mutation of that residue rendered cells insensitive to TNFα repression of β-catenin signaling. Analogous to results shown by Clevers et. al., β-catenin, that has both the residues de-phosphorylated (Ser37 and Thr41), is transcriptionally 'active' and that TNFα, Edar and activated IKKs significantly reduce this 'active' pool in the nucleus, which corresponds to the decrease in luciferase activity [234]. Phosphorylation sites that include consensus sequence DSGXXS and Ser45 regulate β-catenin activity after cytokine stimulation. Immunohistochemistry using IKKα (-/-) mice fibroblast cells show that β-catenin is up-regulated within the nucleus. A further experiment to confirm this, includes the use of the de-phosphorylated antibody in IKKα (-/-) and IKKβ (-/-) embryonic skin cells harvested at E14-16 when Edar expression is highest and to determine the localization of β-catenin in these cells. De-phosphorylated β-catenin is expected to be absent from the nucleus since Edar represses β-catenin activity.

Our results also conclude that NFκB is not involved in the cytokine repression of β-catenin activity. Thus does not seem to be direct communication between the two pathways, even though they both utilize the IKK complex. There may be other adapter proteins involved in the Edar/IKK pathway. The use of recombinant ectodysplasin would greatly improve the experimental system since over-expression of Edar itself may over-stimulate the cell and create a non-physiological situation. Another approach would be to immunoprecipitate the Edar adapter protein, Edaradd following the addition of the ectodysplasin and probe for β-catenin to identify other proteins that may be found in the complex with IKK. This may address why a complete reversal in response to mutated forms of β-catenin was not observed since it may bind to other adapter proteins through its armadillo repeats. We stated that TNFα and activated IKKs negatively regulate the Wnt signaling pathway and effectively control β-catenin signaling activity within the cell. However, experiments with knockout MEFs have been inconclusive since they do not seem to respond very well to Wnt conditioned media so it is not clear if the IKK complex

plays an active role in the negative regulation of Wnt signaling.

It is challenging to reconcile conflicting the observations related to β-catenin re-localization within the cell and/or degradation through. Immunocytochemistry shows that de-phosphorylated β-catenin disappears from the nucleus (or total cell) when IKKs are activated. However, western analysis performed on cytoplasmic extracts indicate that degradation is not occurring because there is no decrease in the de-phosphorylated fraction. In fact, there is no change in β-catenin protein levels at all. Taking into consideration all the immunocytochemical results observed so far, a possible explanation would be that activated IKKs phosphorylate Ser45 on the β-catenin N-terminus and this increases the affinity for further phosphorylation on Ser33,37 and Thr41. A point to note is that when de-phosphorylated β-catenin is detected within the nucleus, other residues may still be phosphorylated. It is possible that when β-catenin is in the nucleus, part of the protein, especially the N-terminus is masked by nuclear material, such as chromatin and the antibody used may not be able to detect the presence of phosphorylated β-catenin protein. When these residues are phosphorylated, it is possible that β-catenin is targeted for degradation either in the nucleus itself or after translocation into the cytoplasm. However, it is likely that the fraction of de-phosphorylated β-catenin that is transcriptionally active is small, relative to the total pools of β-catenin within the cell; this small change may not detected via western analysis. The best experiment to prove this would be to harvest the nuclear and membrane fractions of SW480 cells after treatment with TNFα and determine the levels of de-phosphorylated β-catenin protein. The de-phosphorylated β-catenin fraction in the nucleus is expected to decrease, indicating that de-phosphorylated β-catenin is being degraded. This should correspond to the increase in the phosphorylated form of β-catenin in cytoplasm as observed earlier in chapter three. These initial experiments were carried out but there were technical difficulties in obtaining pure nuclear extracts. In Sw480 cells, even a small (<1%) contamination from the cytoplasmic or membrane pool significantly disturbs the results. Another method not yet utilized might be to isolate the nuclear fraction and perform use high salt buffer to extract the nuclear proteins that are associated with chromatin..

Recent studies have suggested an additional pathway whereby Wnt and cytokine signaling may interact. Studies in mice have shown that Wnt 6 is able to regulate the expression of ectodysplasin (Eda) [240]. Laurikkala et al. have shown that the expression of Edar is induced by activin A and its signaling from mesenchyme may induce the expression of Edar in the epithelial signaling centers, thus making them responsive to Wnt-induced Eda from the nearby ectoderm [241]. Moreover, Eda expression is down-regulated in LEF-1 mutant mice, suggesting that signaling by ectodysplasin is regulated by LEF-1-mediated Wnt signals. It is important to note that the phenotype of ectodermal dysplasia syndrome together with the fact that mutation in humans and mice cause loss of gene function strongly suggest that ectodysplasin and Edar promote cell survival rather than apoptosis [164;242]. Also, expression of Eda promotes cell adhesion to the extracellular matrix, which is consistent with a role of this protein in epithelial-mesenchymal interactions regulating the development of ectodermal appendages. Taking into account all these observations, it is not surprising that TNFα and Edar signaling may be differentially regulated.

Thus the results in this study show that the addition of TNFα and Edar result in the activation of IKKα and IKKβ. This may translocate into the nucleus resulting in the phosphorylation of β-catenin on Ser45. In turn, this results in its translocation out of the nucleus where further phosphorylation occurs on residues Ser33, Ser37 and Thr41. This may target it for degradation via other F-box proteins in reducing β-catenin signaling activity.

It is interesting to observe how nature has tried to conserve the use of its resources resulting in the same protein having different roles in development and cell homeostasis. β-catenin is involved in cell-adhesion and is also involved in cell signaling. It is possible that this evolved through a result of the gastrulation process where cells are multiplying but moving to arrange themselves and are brought into new positions in embryogenesis. When cells are going through gastrulation, β-catenin protein levels in the cells are high, indicating that it is present at the membrane to allow for cell adhesion and also present in the nucleus to stimulate growth promoting genes, like cyclin D1 and c-myc. Expression levels of β-catenin drop when cells have completed this process.

References:

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Manuscript is in preparation.